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TANDEMLY REPETITIVE DNA IN THE KARYOTYPIC AND PHYLOGENETIC EVOLUTION OF *CERVIDAE* SPECIES

BY

CHARLES LEE

©

A Thesis

submitted to the Faculty of Gradute Studies and Research

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

IN

EXPERIMENTAL PATHOLOGY

DEPARTMENT OF LABORATORY MEDICINE AND PATHOLOGY

EDMONTON, ALBERTA

FALL, 1993

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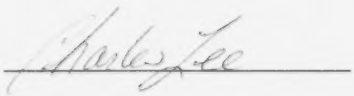

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'*The eukaryote chromosome is a box of surprises.*'

A Lima-de-Faria. (1983) *Molecular Evolution and Organization of the Chromosome*, Elsevier Science Publishers B.V.

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The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **TANDEMLY REPETITIVE DNA IN THE KARYOTYPIC AND PHYLOGENETIC EVOLUTION OF *CERVIDAE* SPECIES** submitted by **CHARLES LEE** in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE** in **EXPERIMENTAL PATHOLOGY**.

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Date: **October 4, 1993**

TO MY DEAR WIFE, CECILIA,
MY LOVING FAMILY: MOM, DAD, JAMES & JENNY
AND MY LORD, JESUS CHRIST.

... THANKS FOR SEEING ME THROUGH.

ABSTRACT

This thesis employs the use of tandemly repetitive DNA in elucidating the karyotypic and phylogenetic evolution of several deer species in the family, *Cervidae*. The first study (Chapter II) focuses on the two closely related deer species which exhibit extremely different karyotypes: the Indian muntjac (*Muntiacus muntjak vaginalis*), 2n=6 chromosomes in females and 2n=7 chromosomes in males and the Chinese muntjac (*Muntacus reevesi*), 2n=46 chromosomes. It has been suggested that a series of tandem chromosome fusions of ancestral Chinese muntjac-like chromosomes may have occurred in the past to produce the large chromosomes seen in the Indian muntjac karyotype. During this investigation, the tandemly arranged human telomeric DNA sequence, $(TTAGGG)_n$, which has been found to be highly conserved in the telomeres of mammalian species, was localized by fluorescence *in situ* hybridization (FISH) on the metaphase chromosomes of the Indian and Chinese muntjac and the Canadian woodland caribou (2n=70). As expected, positive hybridization signals were observed at the termini of almost every chromosome in all three deer species. In addition to the expected positive hybridization at the termini of each chromosome, non random hybridization signals were observed at certain interstitial sites in chromosomes 1 and 2 of the Indian muntjac. These interstitial telomeric signals are believed to represent remnant ancestral telomeric DNA and correlation of these sites to previously identified regions of interstitial heterochromatin has permitted the elucidation of the chromosome sites where breakage and fusion most likely occurred during the restructuring of the ancestral Chinese muntjac-like chromosomes to form the present day Indian muntjac karyotype.

In the second study (Chapter III), the phylogenetic relationship of the Canadian woodland caribou (*Rangifer tarandus caribou*) in the *Cervidae* family was examined. A 991 bp tandemly repeated centromeric DNA sequence (Rt-Pst3) was isolated and found to be well conserved in the genomes of the several deer species studied. The results of

register size (repeat unit size amplification), hybridization intensity, and DNA sequence homology all suggest that the Canadian woodland caribou, roe deer, white-tailed deer, and mule deer are more closely related to each other than to either the North American elk or the Asian muntjacs. Furthermore, DNA sequencing data demonstrate how the 991 bp repeat unit of the woodland caribou may have been derived from a more ancestral monomer unit of ~800 bp. This would suggest that along with chromosome fusions, the repeat units of certain satellite DNA families in the *Cervidae* family could be expanding in length, during the course of evolution.

ACKNOWLEDGEMENTS

This thesis and the completion of my Master of Science training would not have been possible without the support of a number of people who I would like to acknowledge. First and foremost, I am extremely grateful to my supervisor, Dr. C.C. Lin, for taking care of me during this program. You have always believed in me even when others doubted my abilities and you have taught me to always strive for excellence. I would also like to express my thanks to the members of my supervisory committee, Drs. Patrick Ferreira and John Kuspira for taking the time to patiently read and edit this thesis, and for actually making my "dreaded" defense a most enjoyable experience. Also, I am very thankful to an "old" high school friend and a most talented artist, Mr. Keath Lengle, for producing the magnificent cover drawing for this thesis.

I am very thankful for the many friends that I have made in Dr. Lin's labs. In the Cytogenetics Research lab, Dr. Y.S. Fan, who taught me the *in situ* hybridization technique and Dr. R. Sasi, who taught me general molecular biology techniques, have been most helpful and patient with me. To Mr. Dean Court, I would like to thank you for your smile and humor which has always brightened up the lab and made it a pleasant place to come to, at any time of the day. In the clinical lab, I would like to thank Mrs. Helen Worobec for helping me with far too many last minute deadlines as well as the encouragement and friendships of Fran, Shirley, Penny, Melanie, Carol, Ruth, Lynn, Audrey, Christine, and Susan.

I would also like to thank Dr. Nam Soo Kim for encouraging me into the field of cytogenetics and originally introducing me to Dr. Lin. As well, I am very grateful to Dr. D.B.C. (Bruce) Ritchie who was always eager and willing to go out of his way to help me with most of the molecular biology techniques employed in Chapter III. I would also like to thank friends at the Alberta Place Hotel (Rachelle, Jean, John, Rob, Shelly, Glenn, Dionne, Geoff, Carla, Julie, and Mr. I. Guttman) who have always been supportive of my

studies and have provided me with a quiet place to study during the weekends, not to mention a significant portion of my monthly income. Financial support was also provided by an NSERC grant awarded to Dr. Lin and teaching assistanceships in the Department of Genetics (Dr. R. Bhambhani).

To Mom, Dad, James, Jenny and Cecilia (who became my wife during my M.Sc. program and who has tolerated the mess that I made at home during the writing of this thesis); I dedicate this thesis to you. Finally, I thank my Lord, Jesus Christ for making all of this possible and for opening up this path in my life.

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CHAPTER I

INTRODUCTION

A brief literature review of tandemly repetitive DNA in the context of heterochromatin, centromeres, and telomeres. As well, past investigations into the karyotypic and phylogenetic evolution of deer species are discussed.

Our understanding of the ubiquity of repetitive DNA in higher eukaryote genomes stems from the original reassociation experiments of Waring and Britten (1966). Waring and Britten discovered that the DNA component which is separable from the bulk of mouse genomic DNA by means of cesium chloride gradient centrifugation (Kit, 1961), reassociated more rapidly than the remaining genomic DNA. Surprisingly, the DNA component was even found to reassociate some 15 times more faster than SV40 viral DNA. Waring and Britten reasoned that this DNA component must consist of short, reiterated DNA sequences which are present in numerous copies within the mouse genome (in this case, 10^6 copies / genome). Subsequent studies, including a series of reassociation experiments on several other species (Britten and Kohne, 1968) verified the presence of repetitive DNA in essentially all higher eukaryote genomes.

Although some repetitive DNA sequences are now known to actually be functional genes (eg. histones, ribosomal RNA, families of actin, β-globulin, and immunoglobulin genes; see review by Long and Dawid, 1980), a substantial portion of repetitive DNA is believed not to code for any structural proteins. These repetitive DNA families[1] can either be interspersed among other genomic DNA sequences (eg. LINES[2] and SINES[3]) or tandemly organized in one or more chromosomal regions. The tandemly repetitive DNA, commonly referred to as satellite DNA (Pech *et al.*, 1979)[4], is most often associated with three particular chromosomal regions: constitutive heterochromatin, centromeres, and telomeres.

---

[1] A family of repetitive DNA is a collection of repetitive DNA sequences all of which have the same repeat length size and an identical or similar DNA sequence.

[2] Long interspersed DNA elements (each repeat unit usually being 1 kb or larger)

[3] Short interspersed DNA elements (each repeat unit usually being less than 500 bp in length)

[4] Pech *et al.*(1979) suggested that any tandem repetition of a unit DNA sequence be classified as "satellite" DNA. Satellite DNA does not necessarily indicate that the members of a satellite DNA family are separable by isopycnic centrifugation.

Constitutive Heterochromatin:

Constitutive heterochromatin was first observed and defined by Heitz (1928). Heitz noticed that certain regions of eukaryote chromosomes remained in a condensed state throughout interphase while the remaining regions of the chromosome decondensed. Heitz referred to the condensed chromosome regions as (constitutive) heterochromatin.[5] Certain properties of constitutive heterochromatin include its differential staining, chromosomal localization, and DNA composition.

Differential staining:

Constitutive heterochromatin can be cytologically distinguished from euchromatin on the basis of its differential staining properties. The most common method used to reveal regions of constitutive heterochromatin is C-banding (Pardue and Gall, 1970; Jones, 1970; Arrighi and Hsu, 1971; Sumner, 1972). This technique involves an acid treatment (eg. 0.2 N HCl), hot alkali exposure (eg. 5% barium hydroxide at 50°C), a warm saline solution treatment (eg. 2xSSC) and finally, staining with Giemsa. The mechanism of C-banding is still controversial but is generally believed to involve a preferential extraction of DNA and/or proteins in non-C-band regions (Comings *et al.*, 1973; Pathak and Arrighi, 1973). Furthermore, it is commonly known that some C-bands will not appear under certain C-banding conditions and not all constitutive heterochromatin in an organism will necessarily produce C-bands. Therefore, constitutive heterochromatin is often but not exclusively defined by C-bands.

Another banding technique, referred to as N-banding was originally used to identify nucleolar organizing regions (NORs) in several mammalian species (Matsui and Sasaki, 1973). However, it has also often produced bands which do not correspond to NOR regions, but rather to certain regions of heterochromatin. In various species of

---

[5] Heitz actually defined these regions as *heterochromatin*, but they are now more descriptively referred to as *constitutive heterochromatin* in order to differentiate it from *facultative heterochromatin*.

cereals (Gerlach, 1977; Jewell, 1981; Endo and Gill, 1984; Schlegel and Gill, 1984), *Drosophila* (Pimpinelli *et al.*, 1976; Hagele, 1977) and locusts (Hagele, 1979), N-bands occur consistently in certain heterochromatic regions. N-banding commonly employs successive extractions of chromosome preparations with trichloracetic (5% at 90°C for 30 min) and hydrochloric acids (0.1N at 60°C for 40 min) and is believed to progressively extract nucleic acids and histones (Matsui, 1974). Regions rich in acidic proteins, like the 41 kilodalton non-histone protein isolated by Matsui *et al.* (1986), are believed to be more resistant to these extraction procedures and can subsequently be stained with Giemsa. Alternate methods for N-banding include Funaki *et al.*'s (1975) protocol which consists of a 15 min incubation in a 1M solution of sodium dihydrogen phosphate, pH 4.2 at 96°C and Gagne's (1981) substitution of DNase and RNase for trichloracetic acid produced similar N-banding results. Furthermore, other techniques have also been used to apparently identify distinct classes of heterochromatin, primarily in plants: Feulgen banding (Yamasaki, 1956, 1959; Takehisa, 1968, 1969; Yamasaki, 1971) and Hy-banding (Greilhuber, 1973, 1975). These methods employ the use of a hot hydrochloric acid treatment, prior to Feulgen or aceto-orcein staining.

A number of fluorescent dyes (fluorochromes) can be used to further characterize regions of constitutive heterochromatin according to their DNA composition. For example, fluorochromes such as Quinacrine (Caspersson *et al.*, 1968), Hoechst 33258 (Hilwig and Gropp, 1972), 4', 6-diamidino-2-phenylindole (DAPI) (Schweizer and Nagl, 1976), daunomycin and adriamycin (Lin and van de Sande, 1975) produce bright fluorescent bands at specific AT-rich heterochromatin regions. Alternatively, chromomycin A_3 (Schweizer, 1976), mithramycin (Schnedl *et al.*, 1977), and olivomycin (Van de Sande *et al.*, 1977) are GC specific fluorochromes producing specific bright bands in GC rich heterochromatin regions when subjected to the correct excitation wavelength. The resulting chromosome bands can also be enhanced by counterstaining with non-fluorescent, DNA

binding, base-specific antibiotics. For example, the non-fluorescent and GC specific antibiotic, actinomycin-D, can be used to enhance the resolution of the fluorescent bands produced by the AT specific fluorochrome Hoechst 33258 (Jorgenson *et al.*, 1978). Similarly, the non-fluorescent and AT specific antibiotic, netropsin, has been found to enhance fluorescent bands produced by the GC specific fluorochrome olivomycin (Jorgenson *et al.*, 1978). Using conditions of C-banding and base specific fluorochromes, it is possible to cytologically differentiate specific types of variable heterochromatin (eg. Lin *et al.*, 1982).

The use of restriction endonucleases to characterize regions of constitutive heterochromatin is a relatively new technique. The roots of restriction endonuclease banding can be traced to Jones' (1977) observation that human chromosomes which were first digested with the restriction enzyme, Hae III, generated a G-band-like pattern. Subsequently, Miller *et al.* (1983) and Mezzanotte *et al.* (1983) described the differential staining patterns produced by various restriction endonucleases on human chromosomes. Since then, some applications of these restriction endonuclease banding techniques include determining the number of satellite DNA fractions existing in heterochromatin regions and tracing the phylogenetic origin of each satellite DNA family (for a review, see Bianchi and Bianchi, 1987).

Chromosomal localization:

C- and N-banding are two of the techniques commonly used by cytogeneticists to cytologically locate regions of constitutive heterochromatin in metaphase chromosomes. In mammals, amphibian, and insect chromosomes, positive C-bands are almost always localized to the constitutive heterochromatin near the centromere (centromeric heterochromatin) and telomere (telomeric heterochromatin) regions (Jones, 1970; Pardue and Gall, 1970; Gall *et al.*, 1971; Macgregor and Kezer, 1971; Kurnit and Maio, 1973;

Miklos and Nankivell, 1976; Arnason *et al.*, 1978). Studies of C-banding and N-banding in plants show a fundamental difference in heterochromatin distribution from those seen in animals (eg. Vosa, 1976; Endo and Gill, 1984; Linde-Laursen *et al.*, 1986). C- and N-bands in plant chromosomes can be located at interstitial chromosome sites as well as near centromeres and in telomeres.

DNA composition:

Pardue and Gall (1970) accidently discovered that dark staining centromeric regions of mouse chromosomes corresponded to the localized regions of satellite DNA. These dark staining regions are now known as positive C-bands which represent constitutive heterochromatin harboring satellite DNA. Hsu and Arrighi (1971) further suggested that a given region of constitutive heterochromatin could probably contain a heterogeneity of satellite DNA. Comings (1978) also added that the DNA in constitutive heterochromatin is often highly methylated and transcriptionally inactive. Combined with the fact that tandemly organized DNA has been localized to constitutive heterochromatic regions in a wide variety of species, it is easy to generalize that all regions of constitutive heterochromatin consists exclusively of tandemly repetitive DNA. However, this is not always the case. For example, in the Chinese hamster, constitutive heterochromatin on the sex chromosomes and the centromeric region of chromosome 10 do not appear to contain a high proportion of repetitive DNA (Arrighi *et al.*, 1974). Lima-de-Faria (1983) makes the following statement against the above generalization:

> " It would be naive to expect a direct correlation between heterochromatin and DNA satellites. Heterochromatin is not a 'substance', as some have proposed, neither is it a simple type of organization of the chromosome. Heterochromatin is a chromosome phenotype which is identified by its

characteristic appearance but that results from several molecular arrangements of the chromosome material. It would be inappropriate to expect that heterochromatin would represent at the molecular level a single type of DNA sequence or a single type of arrangement of DNA. The evidence that has accumulated in the last years confirms this point of view."

Centromeric DNA:

The centromere is the crucial structural domain responsible for the proper segregation of chromosomes into daughter cells during mitosis and meiosis. It is cytologically defined as the primary constriction of a metaphase chromosome and its characteristic morphology is apparently due to the lack of a final level of chromatin condensation (Rattner and Lin, 1985, 1987). Attached to the outer surface of the primary constriction is a tri-lamellar disc-like ultrastructure known as the kinetochore. The kinetochore is the site where spindle microtubules attach to move the chromosomes to their respective spindle poles (Rieder *et al.*, 1989). In the kinetochore, a motor protein molecule known as dynein, not only helps the chromosome hold onto the spindle fibers, but actually propels itself to move the chromosome along the spindle apparatus (Vallee, 1990; Hoffman, 1992).

The centromeres of the yeasts, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* have been clearly defined genetically and used in the construction of yeast artificial chromosomes (Clarke, 1990). Generally, the yeast centromere core can be defined as a 220 bp - 250 bp region which is protected from nuclease digestion and contains the DNA elements I, II, and III (Bloom and Carbon, 1982). These three DNA elements seem to be essential for proper centromeric function in the yeast. DNA element I consists of a 9 bp

conserved DNA sequence having the consensus, Pu[6]TCACPuTG. DNA element II does not appear to have a consensus sequence but is usually more than 90% AT-rich and maintains a rather constant length of 78-86 bp of DNA. DNA element III is found adjacent to DNA element II and has the consensus sequence, TGTTTPy[7] TGNTTTCCGAAA (Clarke and Carbon, 1985). This centromeric core is surrounded on both sides by arrays of DNA which are attached to nucleosomes in intervals of 160 bp. The DNA sequences within the centromeric core appear to act independently of the flanking DNA sequences by providing the necessary information for the binding of centromeric components and/or the kinetochore.

Unfortunately, the basic genetic element(s) which are responsible for the function of the mammalian centromere still remains unknown. The formation of the kinetochore at the centromere of mammalian chromosomes is believed to involve interaction of proteins with specific centromeric DNA sequences which may directly or indirectly dictate the assembly of the functional centromere. Of all mammalian systems, the human centromere has been the most thoroughly studied. To date, three classes of tandemly repetitive DNA have been identified in the centromeric regions of human chromosomes and well characterized. These include the classical satellite DNA families I, II, III (Corneo *et al.*, 1968, 1970, 1971)[8] , alpha satellite (alphoid) DNA (Manuelidis and Wu, 1978; Wu and Manuelidis, 1980) , and beta satellite DNA (Agresti *et al.*, 1987; Waye and Willard, 1989a).

Of all of the human centromeric satellite DNAs, alphoid DNA is the only satellite DNA currently found at the centromeric regions of all human chromosomes (Mitchell *et al.*, 1985) constituting approximately 5% of the total human genome (Vissel and Choo, 1987). Thirty three different chromosome-specific alpha satellite subfamilies have been identified

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[6] Pu = purine. Therefore, it can either be an *A* or a *G*.
[7] Py - pyrimidine. Therefore, it can either be a *C* or a *T*.
[8] Human satellite IV was shown to be essentially the same as human satellite III based upon restriction endonuclease periodicity (Mitchell *et al.*, 1979; Frommer *et al.*, 1982)

and combined to create a consensus sequence for this satellite DNA family (Vissel and Choo, 1987; Waye and Willard, 1987; Choo *et al.*, 1991). Each monomer of this DNA family was found to be 171 bp long and generally AT-rich. Furthermore, a centromeric protein, CENP-B, was found to bind to a 17 bp region of the alpha satellite DNA monomer: CTTCGTTGGAAACGGCA (Masumoto *et al.*, 1989). This CENP-B protein was found to be localized to the central domain of the centromere (Earnshaw *et al.*, 1989).

Although alphoid DNA has long been a contender for the functional centromeric DNA sequence, several investigations have suggested otherwise. First, the amount of alpha satellite DNA found in each chromosome has been found to vary from approximately 300 kb in the centromere of the Y chromosome to about 9000 kb in the centromere of human chromosome 7 (Wevrick and Willard, 1989; Jabs *et al.*, 1989). Second, the CENP-B protein was found present in both active and inactive centromeres of dicentric chromosomes (Earnshaw *et al.*, 1989). Finally, a mitotically stable marker chromosome was recently found to be devoid of alphoid satellite DNA and CENP-B proteins (Voullaire *et al.*, 1993). These results tend to suggest the non-essentiality of alphoid DNA in functional centromeres but does not rule out a structural function for this repetitive DNA family.

During the course of my M.Sc. studies, I have been involved in the identification of a new human centromeric satellite DNA sequence (Lin *et al.*, 1993). This new satellite DNA family was localized to the centromeric region of human chromosome 8 and was designated as human gamma-8 satellite DNA. DNA sequencing analysis revealed it to consist of monomer units of 220 bp in length which span a total area of about 430 kb in one chromosome 8 centromere. Further work needs to be done to determine if this new satellite DNA family plays some functional role in cell division.

Telomeric DNA:

The telomere[9] was originally defined by the nobel laureate Hermann J. Muller (1938) over 50 years ago. Muller postulated that the ends of chromosomes played an important role in maintaining the integrity of the chromosome. He termed these chromosome ends "telomeres". It is now known that the telomeres are required for complete replication of linear chromosomes because DNA synthesis only proceeds in a 5' to 3' direction and depends on the presence of a short RNA primer to provide a free 3'-OH group. Without the proper maintenance of the telomeres, each round of chromosome replication would progressively shorten the telomeres until the chromosomes become unstable and the cell dies. Thus, broken chromosome ends that have lost their telomeres are known to fuse to other chromosome ends to prevent subsequent nuclease degradation and mitotic instability (for reviews see Blackburn, 1991; Moyzis, 1991; Kipling and Cooke, 1992).

The realization that a repetitive DNA sequence from the telomeres of *Tetrahymena* chromosomes, $(TTGGGG)_n$, also cross-hybridized to human telomeres (Allshire *et al.*, 1988) led Moyzis *et al.* (1988) to identify the human telomeric repeat array, $(TTAGGG)_n$. There are several reasons why this telomeric DNA repeat is believed to be essential for the formation of a functional telomere. First, this DNA repeat was found to be highly conserved among vertebrate (Meyne *et al.*, 1989) and non-vertebrate species (Moyzis *et al.*, 1988). Second, it has been shown that a chromosome 16 interstitial deletion was stabilized by the formation of a telomeric DNA sequences at the breakage points (Wilkie *et al.*, 1990). Also more recently, the cloned human telomeric DNA has been used to efficiently fragment mammalian chromosomes into acentric chromosome fragments and mitotically stable chromosome truncates (Farr *et al.*, 1992; Barnett *et al.*, 1993).

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[9] from the Greek word: *telos* meaning end and *meros* meaning part

Amplification of Tandemly Repetitive DNA Arrays:

Several explanations have been put forward to account for the amplification and/or deletion of tandemly repetitive DNA. Fry and Salser (1977) suggested a large scale duplication scheme (saltatory replication) as another means of DNA amplification. Smith (1973, 1976, 1978) later proposed a model of unequal crossing over as the driving force for satellite DNA amplification. This model suggested that misalignment of DNA molecules followed by a crossing over event between either sister or nonsister chromatids would result in recombinant DNA molecules that are longer or shorter than either of the original molecules. Walsh (1987) later argued that a counteracting event (intramolecular crossing over) makes it unlikely that unequal crossing over alone is sufficient to expand an array significantly. He therefore proposed that an initial repeat array could be formed by unequal crossing over and then expanded via gene amplification-like processes. Such processes could include a rolling circle replication (Hourcade *et al.*, 1973) which would amplify the DNA of circular plasmids formed by partial deletions of a tandem DNA array, or unproportional DNA replication (Schimke, 1984). Amplified DNA sequences could then be inserted into a chromosome site by homologous recombination. Tandemly repetitive DNA sequences which have been disrupted or lost, could be compensated for by amplification of the remaining DNA repeats by any one or a combination of the above mechanisms.

Amplification of the tandemly repetitive DNA such as the telomeric minisatellite, $(TTAGGG)_n$, is believed to be a result of the action of a ribonucleoprotein known as telomerase (Blackburn, 1990). Telomerase is a type of reverse transcriptase which is composed of both protein and RNA (Greider and Blackburn, 1987). A C-rich RNA molecule (AACCCCAAC) on the telomerase enzyme serves as the template for the synthesis of the G-rich telomeric DNA. This phenomenon has been demonstrated *in vivo* and *in vitro* (Grieder and Blackburn, 1987, 1989; Shippen-Lentz and Blackburn, 1989).

Possible Functional Roles for Tandemly Repetitive DNA:

Since tandemly repetitive DNA is thought to be non-transcribed and in abundance within a genome, it has often being passed off as "junk" DNA with the implications that it is of little significance (Doolittle and Sapienza, 1980; Orgel and Crick, 1980). However, transcribability and uniqueness may not solely dictate the importance of a particular DNA sequence. The telomeric minisatellite is an excellent reminder that although tandemly repetitive DNA may not code for a structural protein, it can provide alternate functions in genome stability, organization, or regulation. Vogt (1990) describes how tandemly repetitive DNA can adopt irregular, locus specific secondary structures. He further suggests that one possible function for some of these locus specific "chromatin folding domains" could be to assist in the transcription regulation of adjacent "protein-coding" sequences. It has also been suggested that chromosome specific subfamilies of human centromeric satellite DNA may permit the recognition between homologous chromosomes for preferential pairing during meiosis (Haaf *et al.*, 1986; Choo *et al.*, 1988). As well, tandemly repetitive DNA have been shown to have a binding affinity to a number of proteins. Examples of this include HMG-like nuclear proteins and the CENP-B centromeric protein to human alpha satellite DNA (Strauss and Varshavsky, 1984; Masumoto *et al.*, 1989) as well as the mouse nuclear protein to the mouse major satellite DNA (Avila *et al.*, 1983).

Wichman *et al.* (1991) further suggested that tandemly repetitive DNA may also be one of the driving forces in the chromosomal rearrangements which lead to speciation (karyotypic evolution). Her postulation is based on the reasoning that chromosomal breakages which occur during karyotypic evolution would need to occur in C-band positive regions (which contain satellite DNA) to have a minimal impact on the euchromatic portions of the genome (Hsu *et al.*, 1978). Hence, repetitive DNA would be an ideal site for the initiation of chromosomal rearrangements which occur concomitant to speciation.

Evolution in the Deer Family:

The largest natural variation of karyotypes can be seen in the deer family, *Cervidae*. Although this mammalian family only has 32-36 living species in the world (Simpson, 1984; Neitzel, 1987), taxonomic classification of certain species in the deer family remains controversial. The diploid chromosome numbers in this family are known to vary from 2n=80 in the Siberian roe (*Capreolus capreolus pygargus*) (Neitzel, 1987) to 2n=6 in the female Indian muntjac[10] (*Mutiacus muntjak vaginalis*) (Wurster and Benirschke, 1970). Furthermore, a close relative of the Indian muntjac, the Chinese muntjac (*Muntiacus reevesi*)[11] has 2n=46 chromosomes all of which are acrocentrics (Wurster and Benirschke, 1967). It was surprising to see two such closely related species exhibiting such extremely diverse karyotypes. This prompted speculations into the evolution of the Asian karyotypes.

Hsu *et al.* (1975) first hypothesized that the chromosomes of the Indian muntjac were derived by repeated centric and tandem chromosome fusions of ancestral Chinese muntjac-like chromosomes. This would imply that chromosome breakages (if any) would have had to occur in the centromeric and/or telomeric heterochromatin of the ancestral acrocentric chromosomes, permitting the fusion of whole chromosome arms with little or no loss of euchromatin. Numerous attempts have been made to obtain evidence for this theory. In 1980, Shi *et al.* discovered that the two species could interbreed to produce viable hybrids. The female hybrid had a karyotype consisting of three large chromosomes from the Indian muntjac father and 23 smaller acrocentric chromosomes from the Chinese muntjac mother. When the G-banded chromosomes of the Chinese muntjac complement were arranged beside the G-banded chromosomes of the Indian muntjac complement in the F_1 hybrids, considerable homology was observed suggesting that multiple tandem

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[10] Incidently, the Indian muntjac is still believed to have the lowest chromosome number among all mammals.

[11] Shi *et al* (1980) demonstrated that these the Indian and Chinese muntjacs could interbreed to produce viable F_1 hybrids.

chromosome fusions could have occurred (Hsu *et al.*, 1975; Elder and Hsu, 1988). However, no interstitial C-bands had been observed to represent remnants of centromeric heterochromatin (Comings, 1971; Lin *et al.*, 1991). DNA studies by Schmidtke *et al.* (1981) and Johnston *et al.* (1982) demonstrated that there was approximately 20% less repetitive DNA in the Indian muntjac cells than in the cells of the Chinese muntjac. This confirmed earlier studies by Wurster and Atkin (1972) that the DNA content of the Indian muntjac cells is approximately 81% of the DNA content found in Chinese muntjac cells. This loss of DNA was believed to be due to the loss of repetitive centromeric DNA during the formation of the Indian muntjac karyotype.

During this time, other groups had begun to isolate centromeric satellite DNA sequences from the Indian muntjac genome (Bogenberger *et al.*, 1982, 1985; Yu *et al.*, 1986) and examine the distribution and organization of the cloned centromeric satellite DNA in both the Indian and Chinese muntjac species. Southern blot analyses with the Indian muntjac satellite DNA probe revealed the presence of highly homologous DNA sequences in the Chinese muntjac genome and when *in situ* hybridization experiments were performed, positive hybridization signals were detected in the centromeric regions of both the Chinese and Indian muntjac chromosomes. However, interstitial hybridization signals could not be conclusively found in the chromosomes of the Indian muntjac.

Other investigators focused on the extremely large centromeric region of the X+3 chromosome of the Indian muntjac. They reasoned that if multiple centric fusions had played a part in the production of this chromosome, one may expect that the large centromere is actually a composite of several ancestral centromeres fused together. Brinkley *et al.* (1984) began a description of the compound kinetochore nature in the X+3 chromosome and observed a non-random clustering of unit kinetochores in the interphase nuclei of the Chinese muntjac. Rattner (1986) later provided more direct evidence for the compound nature of the X+3 kinetochore.

In 1991, Lin *et al.* isolated a highly repetitive centromeric DNA clone from the Chinese muntjac genome and discovered that it produced non-random clusters of hybridization signals at the centromeric and at certain interstitial regions of the Indian muntjac chromosomes by fluorescent *in situ* hybridization. These interstitial hybridization signals were postulated to represent the remnants of centromeric heterochromatin from ancestral Chinese muntjac-like chromosomes which could not be detected by conventional C-banding or isotopic *in situ* hybridization because of the resolution and sensitivity of these techniques. These findings provided new supportive evidence for the proposed tandem chromosome fusion theory.

The Indian and Chinese muntjacs are members of the same genus (*Muntiacus*) and therefore belong to the same subfamily, *Muntiacinae* within the *Cervidae* family. However, most deer species were found to have a diploid chromosome number much higher than that found in the *Muntiacinae* species (for a review see Neitzel, 1987; Fontana and Rubini, 1990). In fact, based on detailed chromosome banding analysis, Neitzel proposed that the hypothetical ancestor of all deer species may have had a chromosome number of 2n=70. Earlier, Taylor *et al.* (1969) examined the karyotypes of 13 different species and hypothesized the ancestral Cervid karyotype as 2n=70-74. To explain the karyotypic diversity found in the deer family, a chromosome fission mechanism was proposed (Todd, 1975). This would entail the general increase in chromosome number from an ancestral diploid number of about 20 chromosomes to 2n=70-74 chromosomes, commonly seen in most deer species today. However, based on the strong evidence for tandem chromosome fusions during the evolution of the Asian muntjacs coupled with White's (1973, 1975) observations that rearrangements occurring in an evolutionary lineage are often of the same type, it is more commonly accepted that chromosome fusions mechanisms are generally responsible for the evolution of deer species. Furthermore, if chromosome fissions occurred during the evolution of deer species, this would require the

reactivation of latent centromeres as well as the addition of new telomeres during each fission process. Although not improbable, this mechanism would be slightly more difficult to achieve.

Bogenberger *et al.* (1982, 1985) and Yu *et al.*'s (1986) discovery that a predominant centromeric satellite DNA from the Indian muntjac was well conserved in the genome of the Chinese muntjac prompted further studies into the conservative nature of centromeric satellite DNA in deer species. Such conservation in centromeric satellite DNA had been previously found in other mammalian species including the CA-SAT DNA in canids (Fanning *et al.*, 1988; Modi *et al.*, 1988)[12] and alpha satellite DNA in primates (Waye and Willard, 1989b). Lima-de-Faria *et al.* (1986) noticed that a cloned 770 bp centromeric clone of the European red deer (*Cervus elaphus*) produced positive hybridization to several deer species studied indicating the presence of highly homologous sequences in the genomes of these deer. As well, they discovered that this centromeric satellite DNA maintained it's "chromosome field"[13] by hybridizing to the centromeric regions of the Indian muntjac chromosomes. Bogenberger (1987) used the cloned IA satellite DNA from the Indian muntjac as a probe in southern blot analyses of several deer species. Again, highly homologous DNA sequences to the centromeric satellite DNA were detected in the genomes of every deer species studied. Furthermore, it was observed that the deer species belonging to the telemetacarpalia division of the *Cervidae* produced a ladder pattern (register) which implied that the homologous satellite DNA was organized in tandemly repetitive units of 1000 bp. The other group of deer species, belonging to the plesiometacarpalia division of *Cervidae*, exhibited an 800 bp register of homologous satellite DNA. Scherthan (1991) and Lin *et al.* (1991) later demonstrated that the

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[12] Incidently, the chromosome evolution in canids has also been shown to be quite extensive (Wayne *et al.*, 1987a, 1987b)

[13] The chromosome field theory was first introduced by Lima-de-Faria (1954). Simply put, this theory suggests that each DNA sequence has an optimal location in the eukaryote chromosome with respect to the telomere and centromere. Further, it hypothesizes that DNA sequences maintained its optimal territory even during extreme reorganization of chromosomes. Therefore, a centromeric DNA sequence would tend to remain at the centromere during chromosomal rearrangements.

conservative nature of a satellite DNA can also be demonstrated at the DNA sequence level. Scherthan cloned a 730 bp satellite DNA, CCsatI, from the roe deer (*Capreolus capreolus capreolus*) and observed its hybridization to the centromeric regions of the Indian and Chinese muntjac chromosomes. In addition, he showed that the DNA sequence of the roe deer satellite clone was approximately 62% homologous to the DNA sequence of the Indian muntjac centromeric DNA clone, IA (Bogenberger *et al.*, 1982, 1985). Lin *et al.* (1991) also discovered that the DNA sequence of the Chinese muntjac centromeric satellite DNA clone, C5, was approximately 80%-85% homologous to the Indian muntjac DNA clones, IA and B1 (Bogenberger *et al.*, 1982, 1985; Yu *et al.*, 1986). This was expected considering the phylogenetic relatedness between the two muntjac species compared to the phylogenetic relatedness between the Indian muntjac and the roe deer, a species in the subfamily *Odocoileinae*. Therefore, it would seem to be possible to use conserved centromeric satellite DNA to determine the phylogenetic relatedness between various deer species. Such genetic studies would assist in clarifying the taxonomic classifications of several deer species which are still in dispute. As well, it would provide invaluable insights into delineating the chromosomal and phylogenetic evolution of this mammalian family.

Scope of this Thesis:

In this thesis, I have used tandemly repetitive DNA to elucidate the karyotypic and phylogenetic evolution of several deer species. In the first study (chapter II), I have focused on further examining the karyotypic evolution of the Asian muntjacs. By using the highly conserved telomeric DNA probe $(TTAGGG)_n$, I have identified telomeric DNA sequences at the termini of the Indian and Chinese muntjac chromosomes. In addition, several interstitial sites of telomeric DNA were found in chromosomes 1 and 2 of the

Indian muntjac. It is believed that these interstitial sites represent the remnants of telomeric DNA from the ancestral Chinese muntjac-like chromosomes. More interestingly, when these interstitial sites of telomeric DNA were found to correspond to certain interstitial heterochromatin regions (Lin *et al.*, 1991), making it possible to determine where breakage occurred in the ancestral muntjac chromosomes during the karyotypic evolution of the Indian muntjac.

In the second study (chapter III), I attempted to study the conservation and evolution of a centromeric satellite DNA from the Canadian woodland caribou (*Rangifer tarandus caribou*) genome. The reason why I decided to study the woodland caribou, a subspecies of reindeer (*Rangifer tarandus*), are several fold. First, despite exhaustive morphological and paleontological information, considerable discrepancy still remains in the classification of the reindeer in the *Cervidae* family. Some investigators have placed this species into the subfamily, *Odocoileinae* (Simpson, 1984) while others prefer to place them into a separate subfamily, *Rangiferlane* (Neitzel, 1987). The karyotype of this species was also found to be quite unique in that it had 2n=70 chromosomes, similar to that of the hypothetical Cervid ancestor (Taylor *et al.*, 1969; Neitzel, 1982, 1987), which harbored extremely large sex chromosomes (that are not usually seen in any other deer species). Moreover, to our knowledge, the karyotype of this subspecies of reindeer, which is believed to have maintained an eco-genetic isolation for as long as 2.5 million years (Banfield, 1961), has not yet been reported. Therefore, to resolve the phylogenetic relationship of the reindeer in the deer family, we have isolated and characterized a tandemly repetitive centromeric DNA sequence from the woodland caribou, studied its genomic organization in several deer species, and compared its DNA sequence with the DNA sequences of previously identified centromeric satellite DNA from other deer species.

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CHAPTER II

INTERSTITIAL LOCALIZATION OF TELOMERIC DNA SEQUENCES IN THE INDIAN MUNTJAC CHROMOSOMES: IMPLICATIONS IN THE KARYOTYPIC EVOLUTION OF THE ASIAN MUNTJACS

A version of this chapter has been published:

Lee C, Sasi R, Lin CC. (1993) Interstitial localization of telomeric DNA sequences in the Indian muntjac chromosomes: further evidence for tandem chromosome fusions in the karyotypic evolution of the Asian muntjacs. *Cytogenetics and Cell Genetics*. 63: 156-159.

Introduction:

Mammalian genomes are very complex and yet are highly organized and generally quite stable. For example, species of great apes that have diverged 20 - 30 million years ago appear to have similar karyotypes with respect to chromosome number and banding pattern (Turleau and de Grouchy, 1979). It was therefore quite astonishing to observe that within the *Cervidae* family, two closely related species which may have shared a common ancestor only 3 - 10 million years ago (Schmidtke *et al.*, 1981), exhibited extremely different karyotypes: the Indian muntjac (*Muntiacus muntjak vaginalis*), 2n=6 in females and 2n=7 in males (Wurster and Benirschke, 1970), and the Chinese muntjac (*Muntiacus reevesi*), 2n=46 (Wurster and Benirschke, 1967).

The chromosomes of the Indian muntjac are much larger than the Chinese muntjac chromosomes and G-banding patterns suggest that a series of tandem chromosome fusions may have occurred in the genome of a Chinese muntjac-like ancestral species to produce the Indian muntjac chromosomes (Shi *et al.*, 1980; Neitzel *et al.*, 1986; Elder and Hsu, 1988). The euchromatic chromosome arms participating in these fusions would remain relatively intact if breakages and reunions occurred at or close to the centromeric regions and/or the telomeric regions of the ancestral acrocentric chromosomes. A DNA sequence from the centromeric heterochromatin of the Chinese muntjac was recently isolated and found to localize to interstitial sites in the Indian muntjac chromosomes, as well as to the centromeric regions (Lin *et al.*, 1991), providing new supportive evidence for the tandem chromosome fusion theory. In this chapter, the highly conserved human telomeric DNA sequence $(TTAGGG)_n$ (Moyzis *et al.* 1988) is found to be located in the telomeric regions of the *Cervidae* species studied, as well as interstitially, at certain non-random sites in the chromosome arms of the Indian muntjac. This information further elucidates the karyotypic evolution of the Asian muntjacs.

Materials and Methods:

Cell Culture. Fibroblast cells of a male Indian muntjac (*Muntiacus muntjak vaginalis*), a female Chinese muntjac (*Muntiacus reevesi*), and a female woodland caribou (*Rangifer tarandus caribou*) were grown in MEM medium (GIBCO) enriched with 18% fetal calf serum and penicillin-streptomycin (100 μg/mL). The male Indian muntjac cell line was a kind gift of Dr. T.C. Hsu, Section of Cellular Genetics, the University of Texas M.D. Anderson Hospital and Tumor Institute, Houston, Texas. The female Chinese muntjac cell line was established from a skin biopsy obtained at the Calgary Zoo, Calgary, Alberta, Canada, and the female woodland caribou cell line was established from a skin biopsy provided by Mr. Jack Nolan, Alberta Environmental Centre, Vegreville, Alberta, Canada.

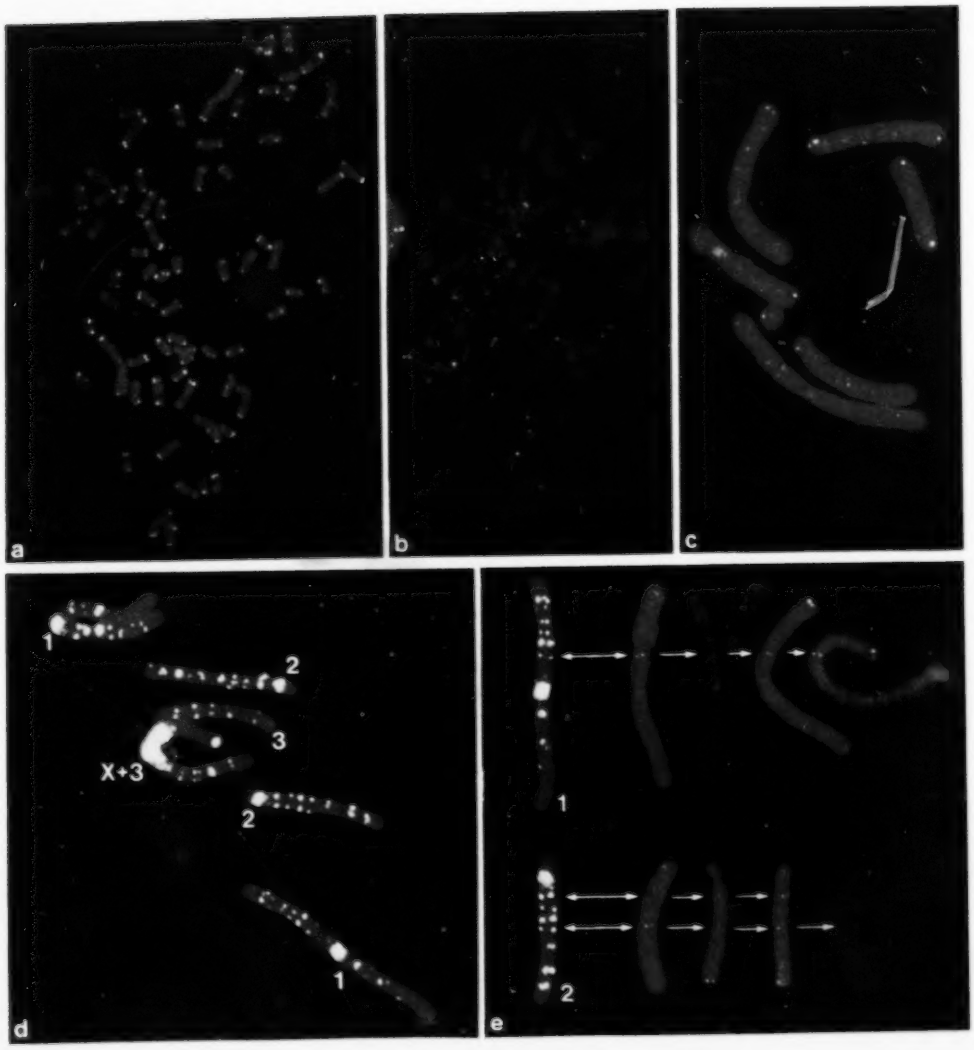
Fluorescence in situ hybridization. For localization of telomeric DNA sequences, the synthetic oligonucleotides (GGGTTA)7 and (TAACCC)7 were labelled with biotin-11-dUTP (ENZO) using terminal deoxynucleotidyl transferase (BRL) as recommended by the manufacturer. Fluorescence *in situ* hybridization was performed based on the protocol of Meyne *et al.* (1990) with slight modifications. Briefly, unaged slides of chromosome preparations were pretreated with RNase A, Proteinase K, and denatured in 70% formamide, 2xSSC (1xSSC: 150 mM NaCl, 15 mM trisodium citrate) for 3 min at 70°C. The probe was then applied at a concentration of 0.5 μg/mL in 25% formamide, 2xSSC, and salmon sperm DNA (100 μg/mL) (Park *et al.*, 1992). Slides were hybridized overnight at 37°C and then washed once in 25% formamide, 2xSSC at 37°C followed by two washes in 2xSSC at 37°C. Immunological detection of hybridization signals was performed as described in Fan *et al.* (1990). Localization of Chinese muntjac's centromeric heterochromatin DNA onto Indian muntjac chromosomes using the C5 DNA probe was performed following the procedures described by Lin *et al.*, 1991.

Results:

Twenty metaphase spreads of *R.tarandus caribou*, fifty five metaphase spreads of the *M. reevesi*, and eighty metaphase spreads of *M.muntjak vaginalis* were analyzed for the presence of hybridization signals with the telomeric oligonucleotide probe. The telomeric repeat was detected on at least one chromosome end of nearly all the chromosomes in the three deer species examined (Fig. II.1, a-c). About 95% of the chromosomes in the examined metaphase spreads of the woodland caribou (2n=70) showed hybridization signals at both chromosome ends. The remaining 5% of the chromosomes had a telomeric signal randomly located on only one of the chromosome ends. In the Chinese muntjac, the telomeric signals were randomly detected on 75% - 80% of the chromosome ends. The hybridization signals at the chromosome termini appeared to have intensities similar to those observed in the termini of the caribou chromosomes. Compared to the telomeric hybridization signals detected in the other two deer species studied, such signals were much smaller in the Indian muntjac chromosomes and consequently were more difficult to detect. Only ~60% of the chromosome ends of the latter species showed hybridization signals. Furthermore, the hybridization signal intensities also appeared to vary randomly at the chromosome termini. However, non-random hybridization signals were observed interstitially on chromosomes 1 and 2 of this species (Fig. II.1, e). About 15% of the metaphase spreads showed these interstitial telomeric signals. Nevertheless, they were consistently present at specific interstitial chromosome sites.

Fluorescence *in situ* hybridization using the C5 centromeric heterochromatin DNA probe to chromosome preparations of the Indian muntjac produced very satisfactory results. Distinct interstitial hybridization signals, as those reported by Lin *et al.* (1991), were clearly demonstrated (Fig. II.1, d). The sites of interstitial telomeric DNA sequences detected earlier appeared to be in close proximity to specific interstitial centromeric heterochromatin sites located in the Indian muntjac chromosomes (Fig. II.1,e). Consistent

Figure II.1. Metaphase spreads and partial karyotypes of three deer species which have been hybridized to the telomeric sequence $(TTAGGG)_n$ and the centromeric heterochromatin DNA probe, C5. Chromosomal localization of telomeric signals to metaphase spreads of **(a)** *Rangifer tarandus caribou*, **(b)** *Muntiacus reevesi*, and **(c)** *M. muntjak vaginalis*. **(d)** A metaphase spread of Indian muntjac chromosomes hybridized to the C5 DNA probe. Distinct interstitial hybridization signals are shown along the arms of all the chromosomes except for the small Y chromosome (outside of this field of view). **(e)** Partial karyotypes of the Indian muntjac using a chromosome 1 and a chromosome 2 from (d). The other chromosome 1s and chromosome 2s were selected from different metaphase spreads which had been hybridized to the telomeric probe. These chromosomes show telomeric signals at the chromosome termini as well as at certain interstitial sites. These interstitial telomeric sites appear to correspond to specific sites of interstitial heterochromatin (indicated by arrows).


a
b
c
1
2
3
X+3
2
1
d
1
2
e

telomeric signals could not be observed at any other site along the Indian muntjac chromosomes, including the centromeric regions.

Discussion:

Localization of the telomeric DNA sequence $(TTAGGG)_n$ in non-telomeric sites has been reported in over half of the vertebrate species studied (Meyne *et al.*, 1990). Two possible mechanisms have been put forward to explain the generation of these observed intrachromosomal telomeric sites: fusion of ancestral chromosomes at telomeric sites or amplification of endogeneous, short $(TTAGGG)_n$ tandem repeats within the chromosome arms. One recent study reports the presence of interstitial telomeric DNA sequences in the chromosomes of all 8 species of North American hylid frogs examined. However, intraspecific variation in the number of these interstitial sites were found between homologous chromosomes of individuals from different populations (Wiley *et al.*, 1992). This observation suggests that aside from chromosome fusions, mechanisms such as unequal crossing over, deletion, and differential amplification, may also be involved in the genesis of certain interstitial telomeric sites.

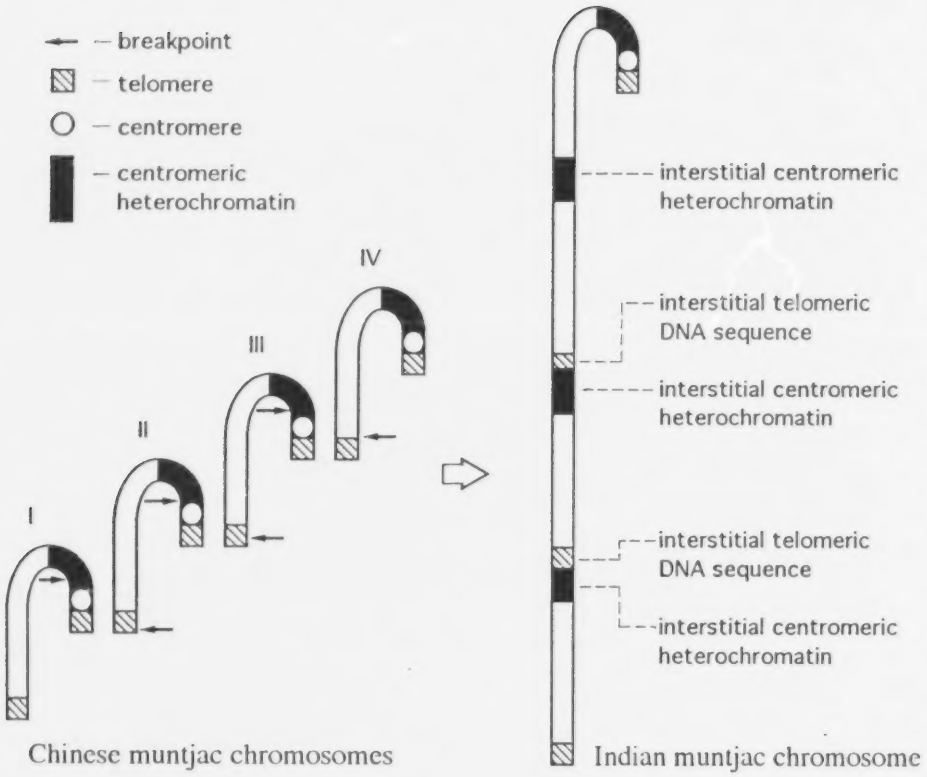
Interstitial telomeric sequences have also been observed at the breakpoint junctions of patients with constitutional chromosome rearrangements (Park *et al.*, 1992). In these cases, rearrangement has occurred to attach a terminal chromosome segment to a recipient chromosome end having a relatively intact telomere. In other cases, such as common reciprocal translocations involving breakpoints proximal of the telomeric region, interstitial telomeric signals were not observed.

The above mentioned investigations demonstrate a variety of mechanisms which could produce interstitial telomeric DNA signals. In the present study, the observation of interstitial telomeric hybridization signals in the Indian muntjac chromosomes is most likely a result of breakage in the centromeric heterochromatin region of an ancestral acrocentric chromosome followed by tandem fusions to the long arm terminus of a second ancestral

acrocentric chromosome, which has retained a substantial portion of its telomere. In this scenario, breakage would have occurred at an extremely distal site on the long arm terminus of the second acrocentric chromosome (Fig. II.2). Association of these chromosome breakage sites could have been achieved by the nature of the repetitive DNA sequences present at the centromeric and telomeric regions (Schmidtke *et al.*, 1981; Bogenberger *et al.*, 1987; Scherthan, 1990). Subsequently, upon fusion of these two chromosomes, one would expect an interstitial telomeric site to be joined to a region of interstitial centromeric heterochromatin. The observed interstitial telomeric signals appeared to correspond to specific sites of interstitial centromeric heterochromatin suggesting that these two DNA sequences are in close proximity to each other. If so, breakage and fusion events could have occurred as described above (Fig II.1, e). Further studies using high resolution *in situ* hybridization to free chromatin (Heng *et al.*, 1992) with dual color fluorescence conjugates may verify the juxtaposition of these two DNA sequences. Chromosome sites having interstitial centromeric heterochromatin but no interstitial telomeric hybridization signal could be a result of breakage in the centromeric region of an ancestral acrocentric chromosome, retaining part of its centromeric heterochromatin, and a more proximal breakage in the telomeric region of the long arm of another ancestral chromosome causing the loss of most, if not all, of its telomeric $(TTAGGG)_n$ repeats. Subsequent fusion of these two ancestral chromosomes would not permit the observation of an interstitial telomeric site at the resulting breakpoint junction.

In some mammalian species, certain satellite DNAs were found to contain a portion of the $(TTAGGG)_n$ repeat (Southern, 1970; Fry and Salser, 1977; Fanning, 1987; Arnason *et al.*, 1988), and the majority of interstitial telomeric sequences were located within or at the margin of constitutive heterochromatin (C-banded) regions (Meyne *et al.*, 1990). However, sequence comparison of the telomeric oligonucleotide with the C5 satellite DNA clone did not reveal any sequence homology indicating that the observed interstitial telomeric hybridization signal is not likely the result of cross hybridization with

Figure II.2. An idiogram depicting chromosome breakages at the centromeric heterochromatin and telomeric regions of four Chinese muntjac-like, ancestral chromosomes followed by tandem fusions, to form an hypothetical Indian muntjac chromosome having interstitial centromeric heterochromatin and interstitial telomeric sites. Close association of the telomeres between ancestral, Chinese muntjac-like chromosomes could have been achieved by a sequence-specific recognition mechanism as illustrated by Scherthan (1990).



Chinese muntjac chromosomes

Indian muntjac chromosome

the C5 DNA sequence. Furthermore, if the interstitial telomeric signals were a result of cross hybridization to the C5 DNA sequence, one would expect to see telomeric hybridization signals at all chromosome sites having substantial amounts of C5-like DNA sequence (including the centromeric region). Since this was not the case, it is believed that the interstitial telomeric signals observed represent remnants of authentic telomeric DNA from ancestral Chinese muntjac-like chromosomes. This finding provides further evidence supporting the tandem chromosome fusion theory in the karyotypic evolution of the Indian muntjac.

For two deer species to be so closely related and morphologically similar, one would expect little or no changes to occur within the euchromatic chromosome arms of the ancestral, Chinese muntjac-like species during the formation of the Indian muntjac chromosomes. This would be possible if chromosomal breakages occurred only within the repetitive DNA sequences of the centromeric or telomeric regions of the ancestral chromosomes. The observed interstitial centromeric heterochromatin and telomeric DNA sites substantiate this notion. Furthermore, one may expect to observe additional interstitial telomeric signals which correspond to the other sites of interstitial centromeric heterochromatin. An explanation for failing to observe such additional signals could be that only very short stretches of telomeric DNA remain at these interstitial chromosome sites. This would produce extremely small hybridization signals which would escape detection with the current resolution of FISH technology.

It is interesting to note that the telomeric hybridization signals on the Indian muntjac chromosomes were smaller than those observed on the chromosomes of the other deer species in this study. Similar findings have also been reported by other investigators (Meyne *et al.*, 1990; Scherthan, 1990). There is a possibility that the Indian muntjac fibroblast cell line used in all of these studies originated from the same source. Further studies using a newly established cell line from another Indian muntjac animal, as

suggested by Meyne *et al.* (1990), may be useful in determining whether this characteristic is actually species specific.

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CHAPTER III

CONSERVATION OF A TANDEMLY REPETITIVE, CENTROMERIC DNA SEQUENCE FROM THE CANADIAN WOODLAND CARIBOU: IMPLICATIONS IN THE PHYLOGENETIC EVOLUTION OF DEER

A version of this chapter has been submitted for publication:

Lee C, Ritchie DBC, Lin CC. (1994) A tandemly repetitive DNA sequence in the centromere of the Canadian woodland caribou (*Rangifer tarandus caribou*): Conservation of centromeric satellite DNA during the evolution of deer species. *Chromosome Research* (Submitted)

Introduction

The family *Cervidae* presently contains 32-36 living deer species (Simpson, 1984; Neitzel, 1987) and exhibits the largest variation in chromosome number observed among all mammalian families. The diploid chromosome number varies from 2n=80 in the Siberian roe (*Capreolus capreolus pygargus*)(Neitzel, 1987) to 2n=6 in the female Indian muntjac (*Muntiacus muntjak vaginalis*)(Wurster and Benirshke, 1970). This diversification in chromosome number has been attributed to rapid karyotypic evolution occurring concomitant to speciation in the form of chromosomal fusions (Shi *et al.*, 1980; Lima-de-Faria *et al.*, 1986; Bogenberger *et al.*., 1987; Elder and Hsu, 1988; Lin *et al.*, 1991; Scherthan *et al.*, 1991; Lee *et al.*, 1993). In fact, detailed chromosome banding pattern analysis of the karyotypes of a large number of deer species have suggested that a common *Cervidae* ancestor may have had a diploid chromosome number of 2n=70 (Neitzel, 1982; 1987).

Despite exhaustive morphological and paleontological information, considerable discrepancy still remains in the classification of species in this family. Extensive chromosome banding analyses (Neitzel, 1987; Fontana and Rubini, 1990), protein electrophoretic variations (Baccus *et al.*, 1983; Emerson and Tate, 1993), mitochondrial DNA sequence analyses (Miyamoto *et al.*, 1990), and satellite DNA (Lima de Faria *et al.*, 1986; Bogenberger *et al.*, 1987) have all been used to further determine phylogenetic relationships among species. Nevertheless, there appears to be no unanimous agreement on any given taxonomic classification for the *Cervidae* family.

One species of deer, the reindeer (*Rangifer tarandus*), has been placed in the subfamily *Odocoileinae* by some investigators (Simpson, 1984) or on its own into a separate subfamily, *Rangiferlane* (Neitzel , 1987). The karyotype of this species is quite intriguing in that it has a chromosome number of 2n=70 (Amrud and Nes, 1966), similar to the hypothetical chromosome number of the ancestral *Cervidae* species and harbours extremely large sex chromosomes. The metacentric X chromosome has been estimated to

have a length of about 9% of the total reindeer complement and the acrocentric Y chromosome is believed to be the largest Y chromosome in all deer species. Large blocks of constitutive heterochromatin have also been found in the long arms of both sex chromosomes (Fraccaro *et al.*, 1968; Gripenberg, 1984).

The reindeer is believed to have arrived in North America during the late Pleistocene period (Kurten, 1971) where they are now commonly referred to as caribou. A subspecies of caribou, the Canadian woodland caribou (*Rangifer tarandus caribou*), is believed to have survived the Wisconsin glaciation in refugia south of the continental ice sheet. Since then, it has maintained an eco-genetic isolation for a period of as long as 2.5 million years (Banfield, 1961). Studies on the genetic variation in transferrin also suggest that the genetic makeup of the present day Canadian woodland caribou is representative of the genome of its progenitors (Roed et al., 1991).

Because of its unique karyotype, ecogenetic isolation, and uncertain taxonomic classification, we have investigated the conservation of centromeric satellite DNA to determine the phylogenetic relationship of the woodland caribou in the *Cervidae* family. Thus, a 991 bp centromeric satellite DNA fragment was isolated from the Canadian woodland caribou genome and designated as Rt-Pst3. In addition to investigating the genomic organization of this 991 bp satellite DNA in several deer species, we have also been able to compare its DNA sequence to previously identified centromeric satellite DNAs of the Asian muntjacs (Bogenberger *et al.*, 1985; Yu *et al.*, 1986; Lin *et al.*, 1991) and the roe deer (Scherthan, 1991). The results suggest that the caribou is more closely related to the roe deer, white-tailed deer, and mule deer than to either the North American elk or Asian muntjacs. Furthermore, the data presented suggest that large repeat units (eg. ~1000 bp) of tandemly organized centromeric satellite DNA in some deer species could have evolved from a more ancestral monomer unit of ~800 bp.

Materials and Methods

Specimens

For total genomic caribou DNA, a fresh kidney sample from a female woodland caribou (*Rangifer tarandus caribou*) was kindly provided by Ms. Margo Pivas and Mr. Bob McClynont, Forensics Department, Alberta Fish and Wildlife, Edmonton, Alberta, Canada. For caribou chromosome preparations, fibroblast cultures were established from skin biopsies of both male and female woodland caribou which were provided by Mr. Jack Nolan, Alberta Environmental Centre, Vegreville, Alberta, Canada. Muntjac DNA and chromosomes were prepared from a male Indian muntjac (*Muntiacus muntjak vaginalis*) cell line originally established by Dr. T.C. Hsu, Section of Cellular Genetics, the University of Texas, M.D. Anderson Hospital and Tumor Institute, Houston, Texas, and a female Chinese muntjac (*Muntiacus reevesi*) cell line which was established from a skin biopsy obtained from the Calgary Zoo, Calgary, Alberta, Canada. The genomic DNA of frog, crane, rabbit, dog, horse, pig, goat, cattle, white tailed deer, mule deer, and elk, that were used for the zooblot study, were previously prepared from blood samples provided to our laboratory.

Cloning of the Rt-Pst3 fragment

Total genomic DNA was prepared from the Canadian woodland caribou kidney specimen as described by Davies (1986). After complete digestion with the restriction endonuclease, PstI, the DNA was fractionated on a 0.8% agarose gel and a prominent ~1kb ethidium bromide stained band was excised. The DNA fragment was electroeluted into dialysis tubing (McDonnell *et al.*, 1977), ethanol precipitated, and then ligated into pUC 19 plasmid vector. The ligation mixture was used to transform *E. coli* bacteria (strain DH5α) and insert-containing plasmids were identified from minipreps of randomly picked colonies

(Maniatis *et al.*, 1982). One of the recovered recombinant plasmids was designated as Rt-Pst3 and used for the present study.

Southern blot analysis

Ten micrograms of total caribou genomic DNA was digested with various restriction endonucleases and used to layer separate wells of a 0.8% agarose gel. For the zooblot study, 10 µg of genomic DNA from each animal was digested to completion with EcoRI and layered into separate wells of another 0.8% agarose gel. Electrophoresis and Southern blotting onto GeneScreen Plus nylon membranes (New England Nuclear) were carried out as described in Maniatis *et al.* (1982). Nylon membranes were prehybridized in a mixture of 1x SET, 10x Denhardt's solution, 0.4% SDS, denatured sonicated salmon sperm DNA (100 µg/mL), and yeast RNA (100 µg/mL) for 4 hours at 56°C. The prehybridization mixture was replaced with a hybridization mixture of 1x SET, 10x Denhardt's solution, 5% dextran sulfate, 0.4% SDS, denatured sonicated salmon sperm DNA (100 µg/mL), yeast RNA (100 µg/mL), and $2x10^6$ CPM of ^{32}P-dCTP (ICN) labelled probe DNA and incubated for 16 hours in a 56°C shaking water bath. Probe DNA was labelled using a random priming kit (GIBCO/BRL) according to manufacturer's instructions with ^{32}P-dCTP. The filters were then washed to 0.1x SSC, 0.4% SDS and exposed to an X-ray film at -70°C for various times.

DNA sequencing

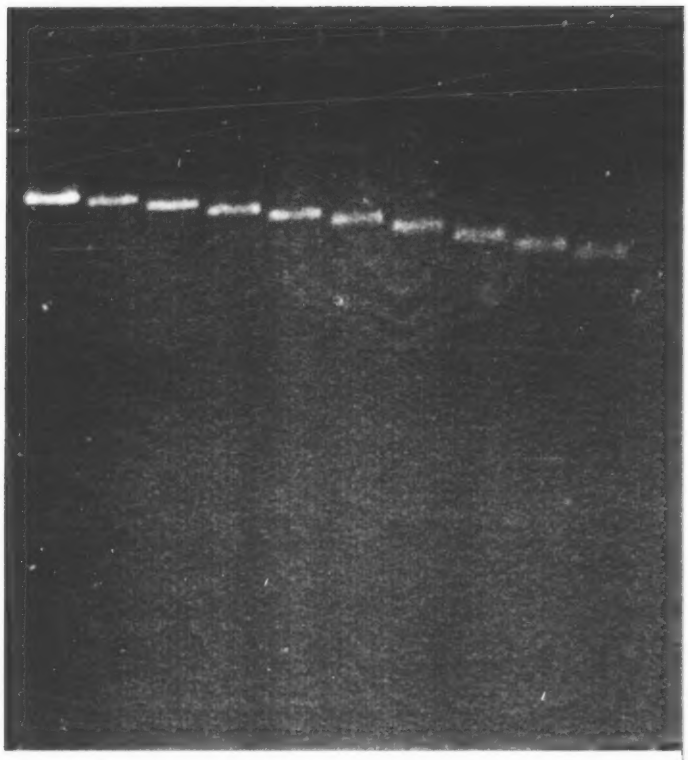
The sequencing strategy of Rt-Pst3 is described in figure III.4A. Subclones of the Rt-Pst3 fragment were produced from XbaI digestions as well as from nested deletions (Henikoff, 1987). Nested deletions were produced from 10 µg of plasmid DNA which was previously digested to completion with both KpnI and SalI, ethanol precipitated, and then resuspended in exonuclease III buffer (15 mM Tris-HCl, pH=8.0, 0.66 mM $MgCl_2$) at a concentration of 0.2 µg/µL. Three hundred units of exonuclease III enzyme

(GIBCO/BRL) were added to the 25 µL of resuspended plasmid DNA which was subsequently incubated in a 37°C water bath. Three microlitre aliquots were removed at 1 min intervals and placed on dry ice into separate microfuge tubes each containing 3 µL of sterile water. Nineteen microlitres of S1 buffer (16 mM sodium acetate, 400 mM NaCl, 1.6 mM $ZnSO_4$, 8% glycerol) containing S1 nuclease (3 units/19 µL) were then added to each microfuge tube and incubated at room temperature for 15 min. Each reaction was stopped with 5 µL of stop buffer (0.8 M Tris-HCl, pH=8.0, 0.02 M EDTA, pH=8.0, 0.08 M $MgCl_2$) followed by a 15 min heat inactivation at 70°C. Eight microlitres of DNA were removed from each nested deletion aliquot and run on a 0.8% agarose gel to determine the extent of each deletion (Fig. III.1). Blunt DNA ends were produced by incubating each DNA aliquot with 1 µL of Klenow DNA polymerase mix (0.2 units/µL of Klenow polymerase in 10 mM Tris-HCl, pH=8.0, 200 mM $MgCl_2$) for 15 min at 37°C. The ligation reactions were finally performed at room temperature for 4 h and each ligation mixture was then used to transform *E.coli* bacteria (strain DH5α). Subclones from the XbaI digestions and nested deletions were sequenced using the dideoxy-chain termination Sequenase™ kit (United States Biochemical Corp.) and the DNA sequence data was compiled and analyzed on a Sequence Editor Software Program (Applied BioSystems).

Copy number estimations

Nuclear DNA isolated from a female woodland caribou kidney sample was serially diluted by one-half to yield a range of concentrations from 10 µg to 156.25 ng. Likewise, control plasmid DNA containing the Rt-Pst3 clone was serially diluted to produce a range of concentrations from 2 µg to 31.25 ng. The diluted DNA samples were subsequently blotted onto a GeneScreen Plus™ nylon membrane using a slot blot apparatus (Tyler Research). Hybridization with ^{32}P-labelled Rt-Pst3 was performed as described for Southern blotting and signal intensities were quantified on a scanning laser densitometer (Ultrascan XL; Pharmacia LKB)

Figure III.1. Agarose gel electrophoresis of 8 μL samples from nested deletion aliquots. Time intervals between each successive deletion is 1 minute.



Chromosome Preparations

All deer cell lines were grown in MEM medium (Gibco) supplemented with 18% fetal calf serum and penicillin (100 units/mL)-streptomycin (100 µg/mL). Cells were harvested after a 1 h incubation with colcemid (0.1 µg/mL), subjected to a 10 min hypotonic treatment (0.075 M KCl at 37°C), and fixed in methanol:acetic acid (3:1).

Fluorescence in situ hybridization.

Fluorescence *in situ* hybridization was performed according to Lin *et al.* (1991) with slight modifications. Briefly, chromosome preparations were pretreated with RNase A (100 µg/mL), proteinase K (0.06 µg/mL), and denatured in 70% formamide and 2x SSC for 3 min at 70°C. The Rt-Pst3 clone was labelled with biotin-11-dUTP (ENZO) by random priming, denatured, and applied at a concentration of 250 ng/mL in 50% formamide, 2x SSC, and salmon sperm DNA (50 µg/mL). Slides were hybridized overnight in a 37°C humid chamber, washed once in 50% formamide and 2x SSC at 37°C, and twice in 2x SSC at 37°C. Probe detection was performed by applying one layer of fluorescein isothiocyanate (FITC) conjugated avidin DCS (5 µg/mL; Vector), a second layer of biotinylated anti-avidin D (12 µg/mL; Vector), and a final layer of FITC conjugated avidin. Slides were then mounted in glycerol containing P-phenylenediamine dihydrochloride (1 mg/mL; Sigma), DAPI (0.8 µg/mL), and PI (0.4 µg/mL). Chromosomes were viewed with a Zeiss Axioskop fluorescent microscope and photographed onto Kodak Gold Plus, ASA 400 film.

Results

Isolation and Southern blot analysis of the Rt-Pst3 clone

Several DNA clones were isolated from the prominent ~1 kb DNA band which was observed against the background smear of ethidium bromide stained PstI digested caribou genomic DNA. One of these clones was designated as Rt-Pst3. To determine the genomic organization of the Rt-Pst3 clone, caribou genomic DNA was digested with several restriction endonucleases, electrophoresed, transferred, and hybridized to ^{32}P-labelled Rt-Pst3 DNA. A type A-like ladder pattern of hybridization bands (Horz and Zachau, 1977) was observed in the PstI genomic digest indicating a ~ 1 kb, tandemly repeated DNA sequence (Fig. III.2). Similar type A-like hybridization patterns with a ~ 1 kb register were also observed in the EcoRI, BamHI, RsaI, HindIII, and KpnI genomic digests. Irregular ladder patterns were observed for XbaI, HpaII, and MspI digested DNA; however, the majority of intense hybridization bands in these digests again showed fragments in a ~ 1 kb periodicity.

Another Southern blot was produced using EcoRI digested amphibian, avian, and mammalian genomic DNAs (including six species of deer). This zooblot was probed with Rt-Pst3 and washed at 64°C and 0.1x SSC. At this stringency, the Rt-Pst3 DNA probe failed to show hybridization to the genomic DNA of the frog, crane, rabbit, dog, horse, and pig (data not shown). A ~ 1.4 kb band was observed in the EcoRI digest of cattle (*Bos taurus*) and three faint bands were observed in the EcoRI digest of goat genomic DNA (Fig. III.3A). The Rt-Pst3 probe hybridized intensely to the genomic DNA of all six species of deer and as expected, the most intense hybridization was found with the caribou genomic DNA. Moreover, very strong hybridization was also observed with the mule deer genomic DNA.

Figure III.2. Genomic Southern blot of EcoRI, PstI, BamHI, XbaI, HpaII, MspI, RsaI, HindIII, and KpnI-digested DNA of a female woodland caribou hybridized with ^{32}P-labelled Rt-Pst3 DNA. Fragment sizes are indicated on the left hand side in multiples of the ~1 kb monomer repeat unit.

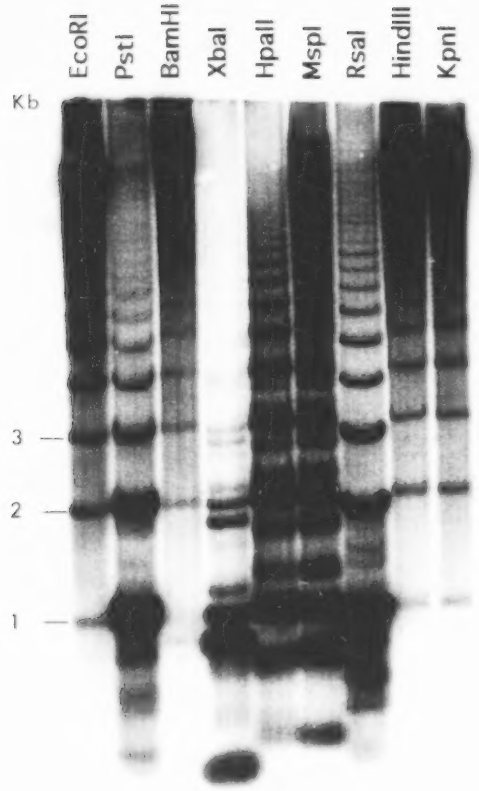
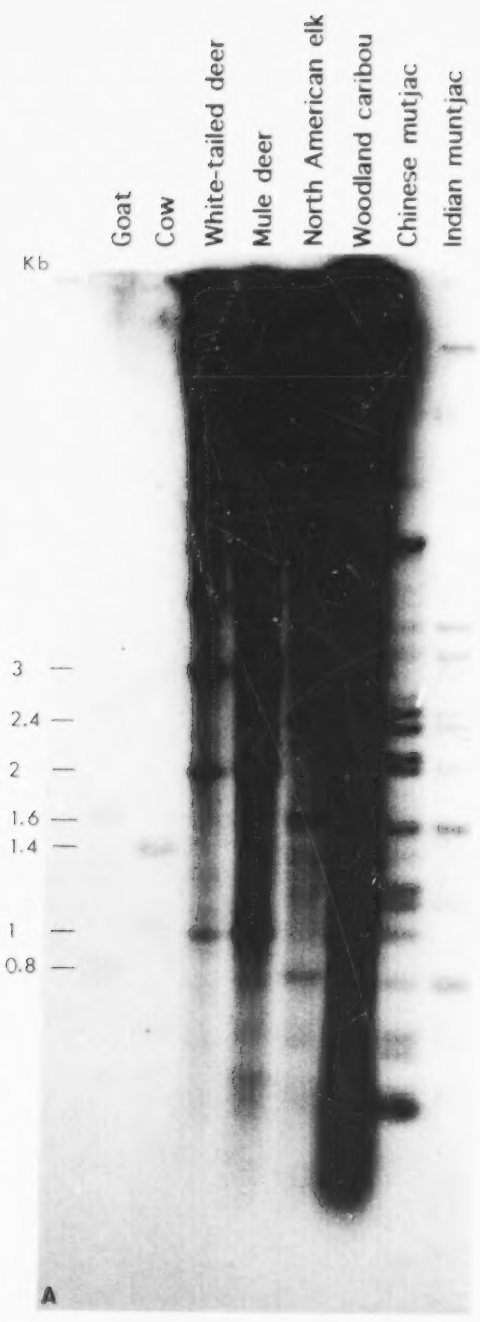
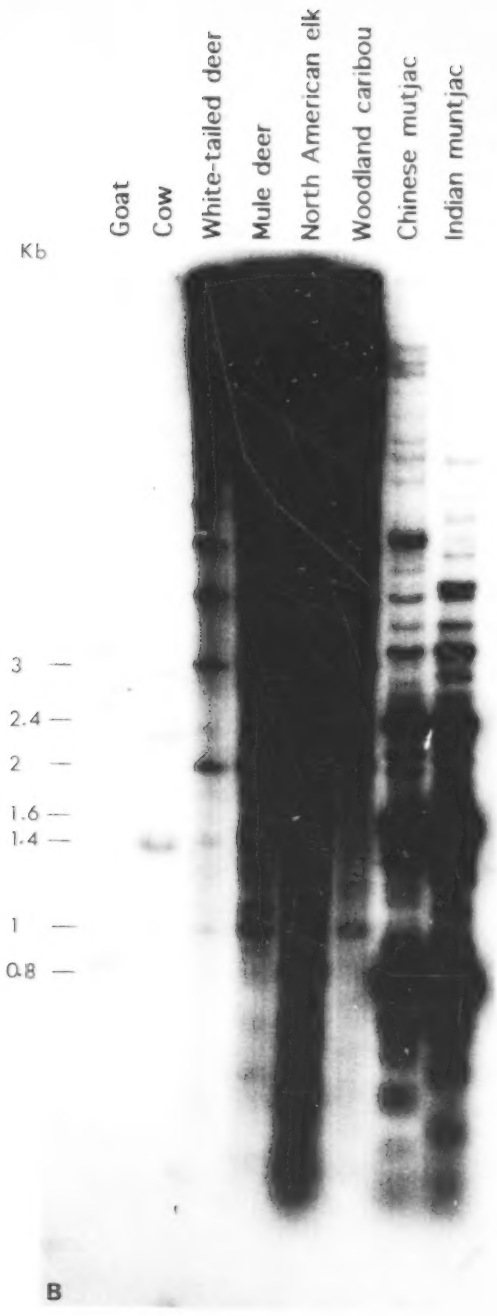

EcoRI
PstI
BamHI
XbaI
HpaII
MspI
RsaI
HindIII
KpnI
Kb
3
2
1

Figure III.3. Genomic zooblot analysis. **A.** Hybridization of the Rt-Pst3 probe to a Southern blot containing goat, cattle, white tailed deer, mule deer, North American elk, woodland caribou, Chinese muntjac and Indian muntjac DNA, all digested with EcoRI. Band sizes are indicated on the left hand side in multiples of 0.8 kb and 1 kb. The 1.4 kb hybridization band generated with cattle DNA is also indicated. **B.** Radioactive Rt-Pst3 DNA was stripped from the blot (in *A*) and re-hybridized with ^{32}P-labelled C5 DNA of the Chinese muntjac. Similar band sizes as those found in *A* are indicated in the left hand side of the panel.


Goat
Cow
White-tailed deer
Mule deer
North American elk
Woodland caribou
Chinese mutjac
Indian muntjac
Kb
3
2.4
2
1.6
1.4
1
0.8
A


Goat
Cow
White-tailed deer
Mule deer
North American elk
Woodland caribou
Chinese mutjac
Indian muntjac
Kb
3
2.4
2
1.6
1.4
1
0.8
B

When hybridization patterns were analyzed, type A-like ladder patterns with bands of ~1 kb periodicities were seen in the EcoRI digested DNA of the white-tailed deer, mule deer, and woodland caribou. A type A-like ladder pattern was also observed in the North American elk, but in a ~0.8 kb register. The genomes of the Asian muntjacs revealed irregular ladder patterns which were almost identical to those previously produced with the roe deer satellite DNA probe, CCsatI (Scherthan, 1991). When the zooblot was stripped of the Rt-Pst3 DNA probe and reprobed with the C5 DNA probe of the Chinese muntjac, intense hybridization was again seen only in the six species of deer. However, the blot showed more of an increased hybridization with the DNA of the North American elk and the Asian muntjacs having dark hybridization bands in ~ 0.8 kb periodicities (Fig. III.3B). Furthermore, a hybridization band of ~ 1.4 kb could be observed with the cattle DNA and a few fainter hybridization bands could be seen with the goat DNA.

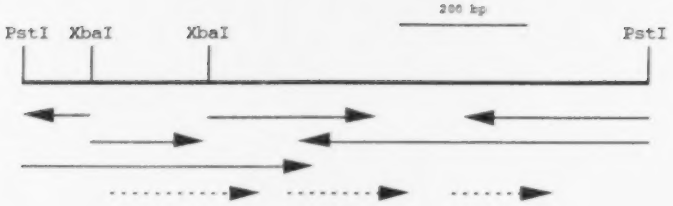
DNA sequencing analysis and comparison

The DNA clone, Rt-Pst3, was sequenced in its entirety using XbaI and nested deletion subclones as described in figure III.4A. The Rt-Pst3 clone was found to be 991 bp long and 54% GC-rich (Fig. III.4C). Moreover, the first 191 bp of the Rt-Pst3 clone was found to be 60% homologous to the last 191 bp (ie. nucleotide positions 800-991)(Fig. III.4D). No other internal repeat could be identified. A HpaII/MspI restriction site (nucleotide positions 229-232) and an RsaI restriction site (nucleotide positions 335-338) were found within the Rt-Pst3 clone as were fourteen motifs of the tetramers CAGG and GAGG.

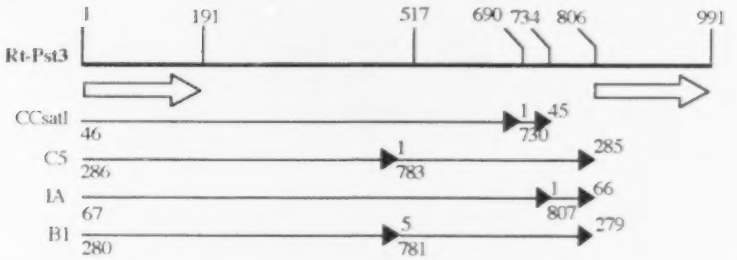
The Rt-Pst3 DNA sequence was then compared to the DNA sequences of previously identified centromeric satellite DNA clones from three other deer species: the roe deer (CCsatI; Scherthan, 1991), Chinese muntjac (C5; Lin *et al.*, 1991), and Indian muntjac (1A and B1; Bogenberger *et al.*, 1985; Yu *et al.*, 1986). Since the largest cloned sequence being compared to the Rt-Pst3 satellite clone was the 807 bp, IA clone of the

Figure III.4. Sequence analysis of the Rt-Pst3 clone and comparison with satellite DNA sequences of other deer species. **A.** Sequencing strategy for the Rt-Pst3 clone. Only restriction sites used for sequencing and subcloning are included. The XbaI subclones were shown as solid arrows whereas the subclones produced by nested deletions are shown as dotted arrows. **B.** Alignment of nucleotide DNA sequences of roe deer satellite DNA clone (CCsatI), Chinese muntjac satellite DNA (C5), and Indian muntjac satellite clones (IA, B1) to the first 800 bp of the caribou satellite DNA clone (Rt-Pst3). The first 191 bp (1-191) and the last 191 bp (800-991) of Rt-Pst3 clone are indicated by empty arrows. Solid arrows represent the length and the direction of satellite DNA sequences of a species alignment with the indicated nucleotide number indicated. **C.** The DNA sequence of Rt-Pst3 and its comparison with four cloned satellite DNA sequences from other deer species. The DNA sequences from each species are aligned (see *B*) to obtain maximum homology with the Rt-Pst3 DNA sequence and the beginning of the proceeding monomer is denoted by a thick horizontal arrow. The restriction sites of HpaII/MspI and RsaI are underlined. Nucleotides of the other four cloned satellite DNA sequence, which are identical to the Rt-Pst3 sequence are indicated by dots. Occasional deletions (-) and insertions (indicated by small arrows and lower case letters) were introduced to improve the alignment. Ambigous bases are denoted as "N". The restriction sites of HpaII and RsaI are underlined. **D.** The DNA sequence comparison of the first 191 bp (1-191) to the last 191 bp (800-991) of the Rt-Pst3 clone demonstrating 60% homology.

A



B



C

```
                      10          20          30          40          50
                       |           |           |           |           |
Rt-Pst3          CTGCAGTGCA  TGCAGAGCTA TTCCGTGA--TC CCATTCA-AAC  AAGTCAGGAN
CCsatI        46.....T...G  ........A. ....T..T--.G ...A...-...  .G..A....G
C5           286T.T..AGC.C  C.AG...-AC .C...A.G-G.. ..--...C...  ..-A.....G
IA            67T.T..AGC..  A.A....-AC .C...A.GCG.. ..--..GC..A  T..A.....G
B1           280T.T..AGCTG  C.AG...-AC .C...A.G-G.. ..--.GGC..T  T..A.....G

                      60          70          80          90          100
                       |           |           |           |           |
Rt-Pst3          CTTCGACTTG  CTTAATGGAT  CTCGAGACAG   TCCCCAAGAA  CACTGTCACC
CCsatI           .C.T..T...  ...G.....A  G.A..TTG..   .....C....  AC.CC....A
C5               TCCT...C.C  G..G..CAC.C  .C..T.TTT.   -AAGGGCC.T  .C.CTCGTTA
IA               TCCTT..G.C  A..G..-.C.C  AC..T.TTT.   GAAGGGCC.T  .C.C.C.GTA
B1               TCCT...GC.  G..G..CAC.C  -GATGTTT..   -GAGGGCC.T  .C.C...GTA

                      110         120         130         140         150
                       |           |           |           |           |
Rt-Pst3          AGTCTAGACG  GACCCTGAGG  TCACCCCAGC  AACGC-AAAGC  AGCTCCATGT
CCsatI           .........A  A..A....T.  .....G...G  ..T.TG...T-  .T...GGG..
C5               .C..G...AT  ATAA.C-..T  ..--.G.C..  ...T.G.G.AA  .A.G-TGA..
IA               .C..G...AT  ATA..CC...  .TC..G.C..  ...T.G.G.AA  .A.GATGA.A
B1               .A..G...AT  ATA..AA...  .AC....C..  ...T.G...A-  .AGCATGA.A
```

```
                    160         170         180         190         200
                     |           |           |           |           |
Rt-Pst3       GCCCCAAATC  AACTCGAGAT  GAGGCCCGAT  TCCCCTGCAT  TGACTCCAGA
CCsatI        AG........  .....T....  T.A....A..  ..........  ..G.......
C5            CT..ACCC..  GC.A......  A.........  .........C  A.CA.G....
IA            CT...CCC..  GC.G....T.  ..........  .T.A.....C  A.CA.G....
B1            CT...C.C..  GC.G......  ......-...  .........C  A.CA.G....

                    210         220         230         240         250
                     |           |           |           |           |
Rt-Pst3       GCCATTCCCC  GTTCCCCATT  CAACACGACC  GGTGGCTCGA  CTTCCTTCAG
CCsatI        A.A.A....T  .A.......C  AT.....T.A  A......T..  ....-..T..
C5            ..A...G.GT  ......A..C  A...C..GAAA .AA.C..T..  T.....-G.T
IA            ..A...G..T  .C.......C  A...C...AA  ..A.C..T..  T......G.TG
B1            .-A...G.GT  CC.......C  -...CT..AA  -.A.C..T..  T......G.TG

                    260         270         280         290         300
                     |           |           |           |           |
Rt-Pst3       GCAACTCCAG  AGATGCCCCG  AGAACACTGT  CCCAAGGTCT  AGAGGAACAC
CCsatI        ......A.C.  .A..T...T.  ...C.CAC..  .TTC....T.  .T......A.
C5            ..-T.A.G..  ..CA.A..GTTCC...-...-..  ----------  ---..-.TC.
IA            ...T.CTT..  AT.GCAT..TT  ..........  .T....-...  .....T.TC.
B1            ...-...... A.T.AA...A.  -.........  .T....-...  .......TC.

                    310         320         330         340         350
                     |           |           |           |           |
Rt-Pst3       CAACTTCAGC  ACAGCAACTC  GAGGAATGCT  CCGTGTACCC  CAAATCGTCT
CCsatI        ...T.....-  ...AG...A.  ......A...  ..A..-..T.  ..T.G.A...
C5            T..AG...CT  T..T....CA  ..-..CA...  T......TTT  ....A.T...
IA            T..AG...CT  T..C....CA  ..-..CA...  ..-....TT.  .G....A...
B1            T..AG...CT  T..C...AC.A ..-..CA... TT......TT.  TG....A...

                    360         370         380         390         400
                     |           |           |           |           |
Rt-Pst3       CGAGATGCGA  GCTGATTCCC  TGGCTTAGGC  TCAAGACGTA  TGCCAACTTC
CCsatI        .A...A.A..  ..........  A..A..CTAT  .G..C.G...  .........T
C5            ....C..A..  .A.....T..  ......CAAA  ......G.A.  ..A.....GT
IA            ....C..A..  .......T..  ......CAAA  ......G.A.  ........GT
B1            ...-------  ......AT..  ......CAAA  ......G.A.  ........CT

                    410         420         430         440         450
                     |           |           |           |           |
Rt-Pst3        CAG-AAGCANC TCAGGAGGAG  GGTTCTCTCA  GCAATAGGTA  TGTGGGAGGG
CCsatI         A.AC.....A. ...A......  CC...C....  C.........  ....A.....
C5           C..-C.....C.  A..A......  .C.....G..  ..TG..C..C  ..GTT.....
IA           C..-C.....C.  A..A......  .C.....G..  ..TG.....C  ..GTT.....
B1           C..-C.....A.  A..A..T...  .T.CT..G..  .CTG..C...  ..GTT.....

                    460         470         480         490         500
                     |           |           |           |           |
Rt-Pst3        ACCCCGAGTT  TGCTGCCTCA  AGGGGAATAT  ACAGCGAGAA  GCCCTAACTT
CCsatI         ....T.....  .T........  ..T...G.GG  .T.C.....T  T....G...A
C5             .GGTT..A..  ...G.....C  ..T.....CG  .ACC.....T  .....G...C
IA           A.GTTT..A..   ...G.....C  ..T.....CG  ..CC.....T  .....G...C
B1             .GGTTA.A..  ...G.....C  ..T.C...C.  ..CC.....T  .....G...C
```

aaccccaggt
ca

```
                    510         520         530         540         550
                     |           |           |           |           |
Rt-Pst3        GAAAGGAGGN  CGGATTTCCT  TGCAGTGGCT  TGAATACAGG  CTCGTCTTTC
CCsatI         ....TA...T  .........C  A..A..A....  .....G....  ..........
C5             ....TC...C  ....A..-..  .-.....A..  ...G.G..A.  ..-.-A-...
IA             ....TC...A  ....A..-.C  .......A..  ...G.G..A.  ..-.GA....
B1             ....TC...C  ....A.T.-.  .......A..  ...G.G..A.  ..-.-A....

                    560         570         580         590         600
                     |           |           |           |           |
Rt-Pst3        ATCTCACAAC  ATGAAGGGAT  CTCTGAATCC  CCTGTGGAGA  CCTTAGAGAA
CCsatI         ....G....G  ..........  G.........  T.........  ..C.......
C5             .........G  ..A.......  G......G..  ....G.....  .AC.......
IA             .........G  ..........  G..G...G..  ...TG.....  .AC....T..
B1             ..A......G  ..........  T......G..  ....GA....  .AC.......

                    610         620         630         640         650
                     |           |           |           |           |
Rt-Pst3        AGCCCTAGGT  CNCTGCCTCA  TTTTGNGGGA  ATGCCTCACA  TCCNCGCTGA
CCsatI         .A....G.T.  .T.C......  .C.G.ACATG  GGC.T.....  ..TCT--...
C5             ........C.  .C......T.  .C.A.A.A.G  CG.....C..  ..AAT-TG..
IA             T.......CA  .AA....CTT  .C.C.ACA.G  CG.....C..  ...AT-TG..
B1             --......C.  .C..-..AAT  ..CA.A.AA.  CC.....C..  ..AAT.AAT-

                    660         670         680         690         700
                     |           |           |           |           |
Rt-Pst3        CACCTCGAGA  GGCACGCGGA  GGTTCATTGC  TTNAAAAGGT  GACGATGCCT
CCsatI         ........C.  ...T.AT...  -.....A...  ..C...-.T.  .GGTTC.AGC
C5             G.G.......  G..A..A....  -.....A..A  ..CC.....A  ..........
IA             ..G..T....  ..A..A....  -.....A..A  ..CC.....A  C.........
B1             ..G.......  ..A..A...T  -CAA..A..A  ..CC.....A  ......CG..

                    710         720         730         740         750
                     |           |           |           |           |
Rt-Pst3        GACTCCTCTT  GAATACTGAT  AGGAATCCCC  AAAATCCCTG  TGGCTACTGG
CCsatI         TC.GTA..CGGG  ..-.C..-T.  ...GT...--  -G.T....
C5             .........G  ...A.TG..C  .........-  .GG.....A.  ....A..A..
IA             .........G  ...A.TG..C  .........-  .GG.....A.  ....A.AA..
B1             ..........  ...A.TG..C  .........-  .GG.....A.  ....A..A..

                    760         770         780         790         800
                     |           |           |           |           |
Rt-Pst3        AAAGGGTCCT  TGGGTGCCCT  GCCTCACCTC  GAGAAGCGTC  CCTTTGGCCC
CCsatI
C5             .T....ATAA  .....CTGTC  .........T T......TT.T  ...A......
IA             ......AA.C  ..C..CTG.C  ..........  ....G.TT.T  ...A......
B1             ........AA  .....CTG..  ..........  ....G.TT.T  ...A.....A

                    810         820         830         840         850
                     |           |           |           |           |
Rt-Pst3        TGCCAAGCCT  CGAAGAGATT  CCTGAGGTGT  CCCTCCGTTA  CTAGACAGGA

                    860         870         880         890         900
                     |           |           |           |           |
Rt-Pst3        GTCCTGACGT  CACTGAAGAA  ACTCGTGTTT  TGAAGGGCCA  TCCCCGCGTA
```

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                      910         920         930         940         950
                       |           |           |           |           |
Rt-Pst3        AGTCGAGAAC  ATACCCCAGG  TCCCCGCCGC  AACTCGAGAA  AAACCATGAG

                      960         970         980         990
                       |           |           |           |
Rt-Pst3        ACTTCCCCCT  AGNGGCGAGA  TGAGGCCCCA  TTCCCCTGCA G
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D

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Rt-Pst3      1 CTGCAGTGCA  T-GCAGAGCT  ATTCCGTGA-  ---TCCCATT  CAA-ACAAGT
Rt-Pst3    800 ....CAA..C  .C.A....--  .....-...G  GTG....-.C  .GTT..T..A

Rt-Pst3     45 CAGGAN-CTT  CGACTTG-CT  TAATGGATCT  CGAGACAGTC  CCCAAG----
Rt-Pst3    846 .....GT.C.  -...G.CA..  G..-.A.A..  ..T.----.T  TTG...GGCC

Rt-Pst3     89 AACACTGTCA  CCAGTCTAGA  -CGGACCCTG  AGGTCACCCC  AGCAACGCAA
Rt-Pst3    890 .T.C.C.-.G  TA....G...  A.AT....C-  .....C..G.  C.....T.G.

Rt-Pst3    138 AGCAGCTCCA  TGTGCC--CC  AAATCAACTC  GAGATGAGGC  CCGATTCCCC
Rt-Pst3    938 -.A.AAA...  ..A.A.TT..  CCC.AGNGG.  ..........  ..C.......

Rt-Pst3    186 TGCATT
Rt-Pst3    987 ....G-
```

Indian muntjac, the first 800 bp of the caribou clone was used for the sequence comparisons (Fig. III.4B). To obtain maximum sequence homology, the first nucleotide of the Rt-Pst3 clone was aligned to nucleotide 46 of the CCsatI DNA sequence, nucleotide 46 of the C5 DNA sequence, nucleotide 67 of the 1A clone, and nucleotide 280 of the B1 satellite DNA clone. After sequence alignment and comparison, the caribou Rt-Pst3 clone was found to have the most DNA sequence homology to the CCsatI DNA clone of the roe deer (73.8%). The Rt-Pst3 clone was also found to have approximately 64.7% homology to the C5 DNA clone of the Chinese muntjac, and 64.8% and 65.6% homology to the 1A and B1 DNA clones respectively of the Indian muntjac. Most mismatches were found to be small base pair substitutions, insertions and deletions. Exceptions to this included a 10 bp insertion in the 1A clone (*aaccccaggt*), an 8 bp duplication (*tcagtgac*) and a 13 bp deletion in the C5 DNA clone, and a 7 bp deletion in the B1 clone (Fig. III.4C). Finally, a higher degree of interspecific sequence homology was found between nucleotide positions 165 to 189, 358 to 376, 485 to 504, 540 to 570, 771 to 800 of the Rt-Pst3 clone.

Copy number estimation.

The copy number of the 991 bp Rt-Pst3 repeat was determined by slot blot hybridization experiments. 1 μg of control plasmid DNA (i.e. ~0.25 ng of Rt-Pst3 DNA) gave a hybridization intensity similar to that of 4.7 μg total genomic DNA. If the genome size of the caribou is similar to that of the Indian and Chinese muntjacs (Lima-de-Faria, 1980), then we can estimate the caribou diploid genome to be at least 5.3×10^9 bp. Using this estimate, it was determined that there are approximately 152, 900 copies of this 991 bp monomer, comprising 5.718% of the caribou genome. This would suggest that an average of 4.6×10^6 bp of the Rt-Pst3 satellite DNA could reside in the centromeric region of each acrocentric caribou autosome.

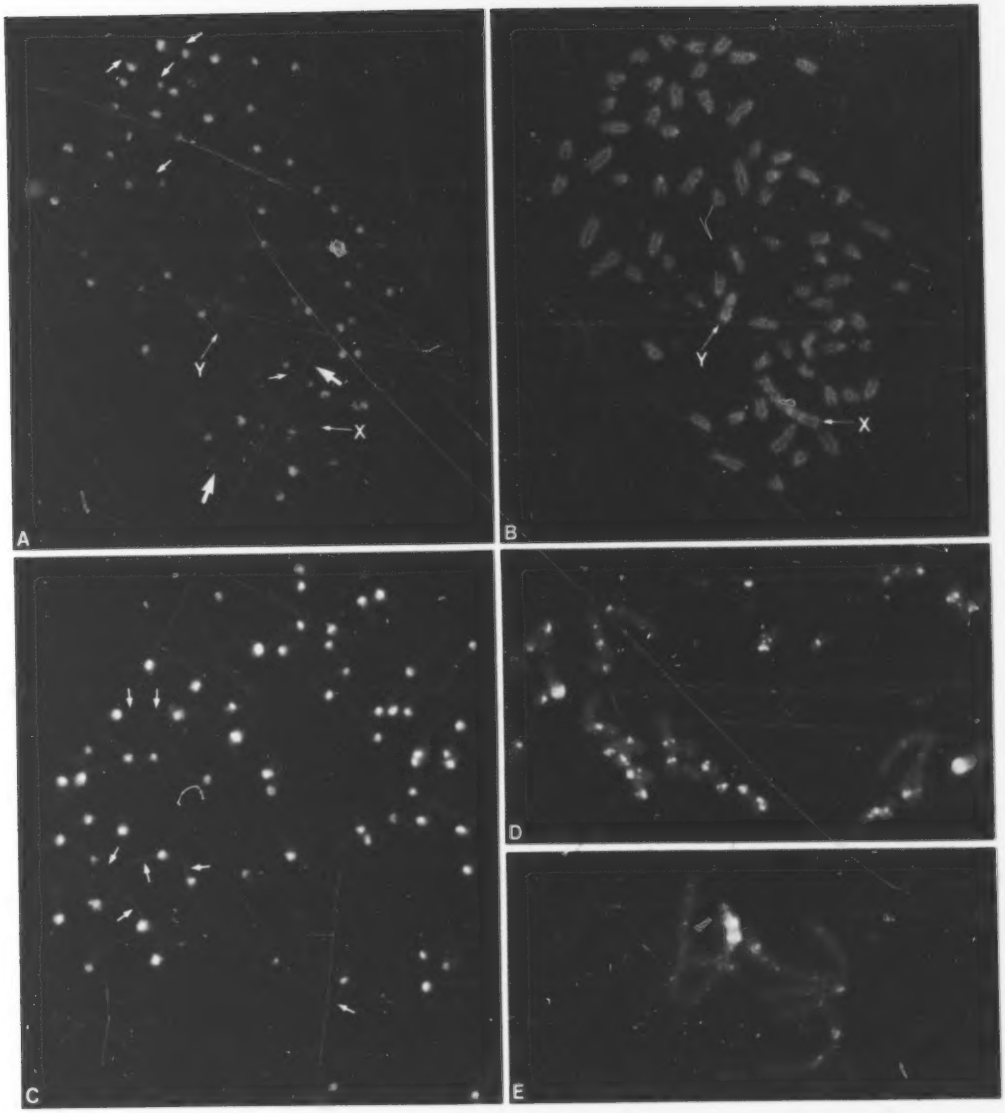
Fluorescence in situ hybridization.

Prior to *in situ* hybridization, chromosome preparations of caribou were examined and found to have a karyotype identical to that of the European reindeer. Metaphase spreads of male caribou were found to have 70 chromosomes consisting of 66 acrocentric and 2 submetacentric autosomes. The metacentric X chromosome was the largest chromosome in the complement and the Y chromosome was among the largest of the acrocentric chromosomes.

The biotinylated DNA probe of Rt-Pst3 was hybridized *in situ* to male and female caribou chromosome preparations. Twenty five metaphases from each sex were examined. Fluorescein isothiocyanate fluorescene (FITC) signals were seen in the centromeric regions of all the acrocentric chromosomes with the exception of the Y chromosome (Fig. III.5A, B). However, fluorescent signals could not be detected in the centromeric regions of any of the biarmed chromosomes including the X chromosome. When the biotinylated Rt-Pst3 DNA probe was hybridized *in situ* to the chromosomes of the Chinese muntjac, fluorescent signals were similarly observed in the centromeric regions of most acrocentric chromosomes (Fig. III.5D). Hybridization of this probe to Indian muntjac chromosomes produced signals at the centromeric, pericentromeric, and several interstitial regions of all chromosomes, except the Y chromosome (Fig. III.5E). The location of the interstitial hybridization signals were similar to those previously reported with the C5 DNA probe (Lin *et al.*, 1991; Lee *et al.*, 1993). Biotinylated C5 DNA was also hybridized to the chromosomes of male and female caribou to determine if any variances in chromosome distribution could be found. The C5 DNA probe revealed similar centromeric distributions in the caribou chromosome preparations, as had been seen with the Rt-Pst3 probe. However, the hybridization signals were slightly weaker (data not shown).

It was also noticed that when routine preparations of caribou chromosomes were hybridized *in situ* with the Rt-Pst3 probe, thin DNA fibers of centromeric satellite DNA were observed emanating from the centromeric regions of some of the acrocentric

Figure III.5. Localization of the Rt-Pst3 satellite DNA sequence to the chromosomes of the woodland caribou, Chinese muntjac and Indian muntjac chromosomes by fluorescent *in situ* hybridization. **A.** A metaphase derived from a fibroblast culture of the male woodland caribou was observed with a filter combination for FITC fluorescence. Strong hybridization signals (yellow-green fluorescence) can be observed in the centromeric region of all acrocentric chromosomes with exception of the Y chromosome (indicated). The two biarmed autosomes (indicated by large arrows) and the X chromosome (indicated) do not show any hybridization signals. Small stretches of chromatin fibers protruding from centromeric heterochromatin regions (indicated by small arrows) can also be observed in some chromosomes. **B.** The same metaphase spread (as *A*) observed with the filter combination for DAPI fluorescence. The centromeric heterochromatin regions of the acrocentric chromosomes show pink color fluorescence as well as the terminal segment on the long arm of chromosome X and a major proximal region on the long arm of the Y chromosome (the X and Y chromosomes are indicated). **C.** A slightly earlier metaphase of a male caribou cell clearly demonstrates stretches of fluorescent chromatin fibers in some acrocentric chromosomes (indicated by small arrows). **D.** A metaphase from a female Chinese muntjac showing hybridization signals, located mainly on the centromeric regions of the acrocentric chromosomes. **E.** A metaphase from a male Indian muntjac showing hybridization signals on the centromeric and interstital regions of the chromosomes. Stronger hybridization signal was observed in the pericentric region of the X+3 chromosome. No hybridization was found in the Y chromosome which is outside of this field of view.


A
Y
X
B
Y
X
C
D
E

chromosomes. The extended chromatin fibers somehow resembled the undercondensed chromatin fibers that protruded from the centromeric regions of mouse chromosomes which had been pretreated with the benzimidazole derivative, Hoechst 33258 and 5-azacytidine (Hilwig and Gropp, 1973; Rattner and Lin, 1987). These chromatin fibers appeared to be more pronounced during early metaphase (Fig. III.5C) and were not detectable under standard DAPI staining.

Discussion:

Lima-de-Faria *et al.* (1984) previously identified a 990 bp HpaII restriction fragment from the European reindeer (*Rangifer tarandus*) and used this uncloned DNA fragment as a probe to Southern blots containing genomic DNA of reindeer and five other deer species. The Southern blot hybridization revealed a type A-like ladder pattern with a register of 990 bp in the HpaII digested genomic DNA of the reindeer. In the present study, an irregular ladder pattern with the major hybridizing bands showing fragments of ~1 kb (990 bp) periodicity was generated from the HpaII digestion of caribou (*Rangifer tarandus caribou*) DNA probed with Rt-Pst3 (Fig. III.2). Since both the 990 bp HpaII reindeer fragment and the 991 bp Rt-Pst3 caribou clone produced a similar register size of ~ 1 kb and a HpaII restriction site was also identified in the Rt-Pst3 clone, it is likely that the HpaII fragment of Lima-de-Faria *et al.* (1984) and the Rt-Pst3 clone belong to the same satellite DNA family. The difference in these hybridization patterns may reflect some slight genetic variations or polymorphisms that have been achieved as a result of the isolation of the Canadian woodland caribou from the present day European reindeer. Indeed, variations in the loci for certain proteins have also been reported between these two species (Baccus *et al.*, 1991). Further Southern hybridization studies using the DNA from both reindeer and caribou should be performed to substantiate this conclusion.

Type A-like hybridization ladder patterns were observed in caribou genomic DNA digested with EcoRI, PstI, BamHI, RsaI, HindIII, and KpnI, when probed with Rt-Pst3 DNA. However, the restriction sites for EcoRI, HindIII, KpnI, and BamHI could not be found within the Rt-Pst3 clone. For such an A-type hybridization ladder pattern to appear with these restriction endonucleases when probed with Rt-Pst3, some repeat units of this DNA family should contain these restriction sites. Numerous potential restriction sites were discovered in the Rt-Pst3 clone which suggest that in certain members of this DNA family, single base pair substitutions, deletions or insertions could have resulted in the production of EcoRI, HindIII, KpnI, or BamHI sites. For example, single base pair substitutions at nucleotide positions 788 or 808 were found to be capable of producing a HindIII site and a single base pair substitution at nucleotide position 334 could produce a KpnI site. Similarly, a BamHI site could be produced by single base pair substitutions at nucleotide positions 572, 724, 756 or a single base pair deletion at nucleotide position 851. Single base pair substitutions at nucleotide positions 180, 241, 328, 515, 536, 579, 633, 715, 727, 952, or single base pair insertions after nucleotide positions 365 or 951 could also produce an EcoRI site.

Interspecific conservation of a satellite DNA can be investigated by means of Southern blot analysis, DNA sequence comparison, and chromosomal localization. When using a satellite DNA as a probe in Southern blot analyses, the observance of positive hybridization bands in the genomic digests of other species demonstrates the existence of restriction DNA fragments that are homologous to the satellite DNA in question. Overall hybridization intensities may provide an indication of the extent of sequence homology between the satellite DNA probe and homologous repetitive DNA in the species' genome. As well, species with similar hybridization patterns and register size are believed to be closely related to each other. For example, hybridization patterns produced by the 990 bp HpaII reindeer probe revealed more similarity between the hybridization patterns of the reindeer, European elk (*Alces alces*), and roe deer (*Capreolus capreolus capreolus*) than

between the hybridization patterns of the reindeer, fallow deer (*Dama dama*), and Asian muntjacs (Lima-de-Faria *et al.*, 1984). This suggested that the reindeer is more closely related to the European elk and roe deer than to the fallow deer or Asian muntjacs. Bogenberger *et al.* (1987) examined the register sizes produced by the genomic DNAs of different deer species when digested with various restriction endonucleases and probed with the Indian muntjac satellite DNA clone, IA. They discovered that the registers of the different deer species studied could be generally classified into one of two sizes: ~800 bp and ~1000 bp. Of the species studied, those belonging to the telemetacarpalia division of the *Cervidae* (ie. *O. dichotomus, O. bezoarticus, M. gouazoubira,* and *C. capreolus pygargus*), predominantly exhibited a ~1000 bp monomer amplification of IA-like satellite DNA. Conversely, among the species studied, those belonging to the Plesiometacarpalia division of the *Cervidae* (ie. *C. duvaucceli, C. porcinus, M. m. vaginalis,* and *M. m. reevesi*) had the majority of their IA-like DNA sequences organized into ~800 bp repeats. The present study also revealed registers of ~ 1000 bp in the caribou, mule deer (*Odocoileus hemionus*), and white-tailed deer (*Odocoileus virginianus*), and registers of ~800 bp in the North American elk (*Cervus elaphus canadensis*) and Asian muntjacs when either the Rt-Pst3 or the C5 DNA probe was used. Based solely on register size, one could conclude that the deer species studied having the ~ 1000 bp satellite DNA register (caribou, mule deer, and white-tailed deer) are closely related and the deer species having the ~800 bp satellite DNA register (ie. the North American elk, subfamily: *Cervinae* and Asian muntjacs, subfamily: *Muntiacinae*) are also closely related. This demonstrates that the deer species, which were palaeontologically classified into the same groups, generally exhibit satellite DNA sequences which are amplified into similar register sizes. Thus, the caribou is believed to be more closely related to the mule deer and white-tailed deer than to either the North American elk or Asian muntjacs. Further analysis showed a more intense hybridization of the Rt-Pst3 DNA clone to the genomes of the caribou and mule deer than to any of the other four deer species in the zooblot. Vice versa, the C5 DNA probe of the

Chinese muntjac was found to produce more intense hybridization patterns to the genomes of the North American elk and Asian muntjacs. Taking this relative hybridization intensity data into account, it can be further suggested that the caribou may be more closely related to the mule deer than to the white-tailed deer. Together these findings tend to agree with the phylogenetic separation of a *Cervinae / Muntiacine* clade from the *Odocoileinae* (Bogenberger *et al.*, 1987; Miyamoto *et al.*, 1990). In addition, our data also show that the caribou appears to belong to the genus *Rangifer*, subfamily: *Odocoileinae* as suggested by Simpson (1984) and Fontana and Rubini (1990).

DNA sequence comparison analyses between the Rt-Pst3 clone and the available satellite DNA clones from other deer species further illustrate the conservative nature of satellite DNA in *Cervidae* species. More DNA sequence homology was found to the CCsatI clone of the roe deer (73.8%) than to the C5 DNA clone of the Chinese muntjac (64.7%) or to the IA and B1 centromeric satellite DNA clones of the Indian muntjac (an average of 64.8%). The sequence homology between the roe deer's CCsatI DNA clone and the Rt-Pst3 monomer can be increased to 76.0% if the first 45 bp of the CC sat I clone is removed from the comparison. When we consider the 80%-85% sequence homology found between the clones of the two very closely related muntjac species (Lin *et al.*, 1991), the satellite DNA sequence homology found between the roe deer, a species in the *Odocoileinae* subfamily and the caribou is quite remarkable. This finding further suggests that the caribou could be classified into the same subfamily, *Odocoileinae*, as the roe deer.

During DNA sequence analysis of the Rt-Pst3 clone of the caribou, it was noticed that the first 191 bp of the 991 bp DNA clone shared 60% homology to the last 191 bp of the clone. This was fascinating because it suggests that this 991 bp monomer clone could have been derived from an original monomeric unit of ~800 bp and that the increased monomer unit size seen in the caribou could reflect the amplification of ~1.2 tandemly repeated ancestral monomers (the original ~800 bp monomeric unit, along with the first 191 bp of the next monomer) prior to homogenization (Southern, 1975; Durfy and Willard,

1989). If this was the case, then other deer species which exhibit a ~1000 bp register of Rt-Pst3-like DNA sequences could have evolved from ancestral species having ~800 bp registers. Scherthan (1991) found that the roe deer (*Capreolus capreolus capreolus*) had a ~2000 bp register. We hypothesize that this ~2000 bp monomer of the roe deer may have been derived from the amplification and subsequent homogenization of 2 tandemly repeated ancestral monomers of 1000 bp. Centromeric satellite DNA having repeat units of 1000 bp have been found in the closely related, Siberian roe deer (*Capreolus capreolus pygargus*) (Bogenberger *et al.*, 1987), which is known to have 2n=76-80 chromosomes including 6-10 B chromosomes (Neitzel, 1987). Based on the high degree of DNA sequence homology found between the Rt-Pst3 and the CCsatI clone, the caribou and the roe deer are believed to be closely related as was previously suggested (Baccus *et al.*, 1983). However, if register size data is also taken into consideration, it is conceivable that the caribou may even be more closely related to the Siberian roe than to the roe deer. DNA sequence analysis of the entire 2 kb monomeric unit from the roe deer and the 1 kb monomeric unit of the Siberian roe deer may shed additional insights into the validity of this hypothesis.

Tetramer CAGG repeats have been found in the Eβ gene of the murine major histocompatibility complex and have been suggested as possible constituents of recombinational hot spots (Steinmetz *et al.*, 1986). Homologous CAGG repeats have also been found in the human hypervariable minisatellite core sequence (Jeffrey *et al.*, 1985). An average of 7 CAGG/GAGG motifs can also be observed in each 220 bp monomeric unit of the recently identified human γ satellite DNA (Lin *et al.*, 1993). Based on random occurrence, only one of these motifs (GAGG / CAGG) are expected in every 128 bp. In the *Cervidae* species, an increased frequency of these motifs have been reported in the centromeric satellite DNA of the Indian muntjac (Bogenberger *et al.*, 1987) and the roe deer (Scherthan, 1991). These motifs can also be found in other previously cloned centromeric satellite DNA sequences of the Indian muntjac (ie. the B1 clone; Yu *et al.*, 1986) and the

C5 clone of the Chinese muntjac (Lin *et al.*, 1991). In this study, 14 such motifs were also discovered in the 991 bp Rt-Pst3 clone of the woodland caribou. The increased number of these motifs in centromeric satellite DNA is believed to have facilitated the karyotypic evolution observed in the deer family (Bogenberger *et al.*, 1987; Scherthan, 1991).

The ability to detect *in situ* hybridization signals, produced by a satellite DNA probe, on the chromosomes of different but related species can also offer more insights into satellite DNA conservation and phylogenetic evolution. In addition to deer species, numerous examples of cloned satellite DNA sequences hybridizing to similar chromosomal locations among related mammalian species have been reported (eg. Modi *et al.*, 1988; Hamilton *et al.*, 1990; Wichman *et al.*, 1991). As well, it has been shown that in some mammalian species, centromeric satellite DNA sequences appear to be more conserved interspecifically than satellite DNA sequences localized to other chromosomal regions in the same species (Fanning et al., 1988). In the present study, the Rt-Pst3 clone was found to be conserved in the centromeric regions of the caribou, Chinese muntjac, and Indian muntjac chromosomes. Although hybridization signals were again observed at interstitial regions of the Indian muntjac chromosomes, these interstitial sites are thought to represent remnants of centromeric DNA from ancestral Chinese muntjac-like chromosomes (Lin *et al.*, 1991; Lee *et al.*, 1993). Hybridization signals with the Rt-Pst3 probe were undetectable in the biarmed chromosomes of the caribou. It has been proposed that biarmed chromosomes could have been derived from Robertsonian fusions of acrocentric chromosomes and that during this process, a majority of the centromeric heterochromatin was lost (Neitzel, 1987). If this is so, this would account for the failure to produce detectable hybridization signals at the centromeric region of these chromosomes. The biarmed chromosomes of the Indian muntjac would then be an exception to this theory.

The stretch of satellite DNA fibers protruding from the centromeric heterochromatin regions of some acrocentric caribou chromosomes, as detected by FISH in this study, is also intriguing. These observations suggest that the completion of the final level of chromatin packing (Rattner and Lin, 1985; 1987) in the centromeric region of some metaphase chromosomes may not always be synchronous. Such undercondensed chromatin fibers could be more prone to breakage and fusion and subsequently facilitate chromosomal rearrangements (eg. Robertsonian translocations), resulting in the karyotypic divergence seen in the deer species. Further *in situ* hybridization studies using cloned centromeric satellite DNA as probes to chromosome preparations from other deer species with large chromosome numbers (eg. 2n=70) may further elucidate this possibility.

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CHAPTER IV

CONCLUSION

A brief recapitulation of the conclusions of the studies presented in chapters II and III.

This thesis has demonstrated how tandemly repetitive DNA can be used in studying the karyotypic and phylogenetic evolution of mammalian species, in this case, deer species. In the first study, the highly conserved telomeric DNA repeat $(TTAGGG)_n$, was used as a probe in fluorescent *in situ* hybridization (FISH) experiments on the chromosomes of the Canadian woodland caribou (*Rangifer tarandus caribou*), the Chinese muntjac (*Muntiacus reevesi*), and the Indian muntjac (*Muntiacus muntjak vaginalis*). Since the human telomeric DNA sequence had already been shown to recognize the telomeres of many species of vertebrates (Meyne *et al.*, 1989), it was not surprising to observe positive hybridization signals at the termini of almost every chromosome in each deer species. What was surprising to see was a number of non-random, interstitial hybridization sites along chromosomes 1 and 2 of the Indian muntjac (Lee *et al.*, 1993). These non random sites were postulated as being remnants of telomeric DNA from ancestral Chinese muntjac-like (acrocentric) chromosomes. Furthermore, the sites of interstitial telomeric DNA were found to correspond well to certain regions of interstitial centromeric heterochromatin previously described by Lin *et al.* (1991). These observations suggest that very distal breakages at the telomeric and centromeric regions of certain ancestral chromosomes must have occurred to leave a significant amount of telomeric DNA and centromeric heterochromatin at these interstitial sites. Other interstitial sites found in the Indian muntjac chromosomes contained centromeric heterochromatin but did not appear to contain telomeric DNA. These chromosome sites could have been produced by a distal breakage in one ancestral acrocentric chromosome followed by fusion to the long arm terminus of a second acrocentric chromosome which had experienced a more proximal breakage in its telomeric region. These observations also affirm the postulation that many of the chromosome fusions, which occurred during the karyotypic evolution of the Indian muntjac, were of a "head to tail" orientation (Hsu *et al* ,1978). Thus, this study provides further evidence strongly supporting the postulation that the chromosomes of the Indian

muntjac evolved from the repeated tandem fusions of ancestral Chinese muntjac-like chromosomes.

In the studies reported on in chapter III, a centromeric satellite DNA fragment was cloned (Rt-Pst3) from the Canadian woodland caribou (*Rangifer tarandus caribou*) to obtain genetic evidence which would provide further insights into the evolution of deer species as well as resolve their taxonomic classification. The conservation of this centromeric satellite DNA sequence in the several deer species examined provided valuable information in determining the phylogenetic relatedness of the caribou (and hence all reindeer) to other deer species. Based on southern blot analyses (register sizes and hybridization intensities), it was demonstrated that the mule deer (*Odocoileus hemionus*) and the white-tailed deer (*Odocoileus virginianus*) had satellite DNA sequences which were highly homologous to the centromeric satellite DNA of the caribou. The homologous DNA sequences of the mule deer and the white-tailed deer further demonstrated a repeat amplification of 1000 bp, similar to that seen in the caribou genome. Less homologous DNA sequences, which were arranged in an 800 bp register, were found in the genomes of the North American elk (*Cervus elaphus canadensis*) and the Asian muntjacs (genus: *Muntiacus*). This supports the notion that the reindeer may not belong to the same subfamily as either the North American elk (*Cervinae*) or the Asian muntjacs (*Muntiacinae*). When the DNA sequence of the caribou satellite DNA clone was compared to the DNA sequences of centromeric satellite clones from the Asian muntjacs (Bogenberger *et al*, 1982, 1985; Yu *et al.*, 1986; Lin *et al.*, 1991) and the roe deer (*Capreolus capreolus capreolus*; Scherthan, 1991), the most DNA sequence homology was observed between the DNA sequences from the caribou and the roe deer. These results strongly indicate that the caribou should be classified in the same subfamily as the roe deer, mule deer, and white-tailed deer; namely the subfamily, *Odocoileinae* (Simpson, 1984; Fontana and Rubini, 1990).

Other observations were made in this study which provide further insights into the evolution of the deer family. First, the 1000 bp monomer of the Rt-Pst3 satellite DNA was hypothesized as being derived from an ancestral 800 bp monomer. This conclusion was based on the fact that the first 191 bp of the Rt-Pst3 clone was 60% homologous to the last 191 bp of the same clone. This could be explained by a selective amplification of 1.2 ancestral monomers to yield, after homogenization, predominantly 1000 bp monomers in the evolved species. Thus evolution in the deer species could favor a general increase in the monomeric unit of this particular centromeric satellite DNA family. This would suggest that the Siberian roe deer (*Capreolus capreolus pygargus*) having 2n=76-80 chromosomes (Neitzel, 1987) and an organization of its homologous satellite DNA into 1000 bp units (Bogenberger *et al.*, 1987), could actually be more ancestral to the European roe deer (*Capreolus capreolus*) which has 2n=70 chromosomes (Armud and Ness, 1966) and homologous centromeric satellite DNA in a 2000 bp register (Scherthan, 1991). Selective amplification of two 1000 bp monomers in the Siberian roe could result in the 2000 bp register seen in the European roe deer.

Another observation made was the stretches of centromeric satellite DNA fibers that protruded from the centromeric heterochromatin regions of some of the caribou acrocentric chromosomes. These stretches of DNA were detected by FISH using the Rt-Pst3 probe. Such undercondensed chromatin fibers may represent an unsynchronized chromatin packing in the centromeric regions of some of the acrocentric chromosomes. As well, these chromatin fibers may be more prone to breakage and subsequent chromosome fusions. Resulting chromosome fusions would be of the Robertsonian type (ie. centromeric fusions). This would concur with Neitzel's (1987) speculations that some Robertsonian fusions may have occurred in *Cervidae* evolution to yield small biarmed chromosomes which are often seen in the deer species.

Consequently, it is believed that chromosome fusions are still the major form of chromosome rearrangement to occur concomitant to the evolution of deer species. The

genetic data from the tandemly repetitive DNA studies provides strong evidence for the classification seen in figure IV.1. I believe that further investigations of satellite DNA in other deer species as well as in other mammalian families could similarly resolve many of the ambiguities which still remain in their respective taxonomic classifications.

Figure IV.1. Taxonomic classification and phylogenetic relationship of the deer species examined in this thesis which is supported by the genetic data of satellite DNA. This classification is similar to that of Simpson (1984) and Fontana and Rubini (1990). Common names, chromosome number, and register sizes of centromeric satellite DNA homolgous to Rt-Pst3 are indicated within the brackets following each species.

Order: ***Artiodactyla***

Family: ***Cervidae***

Subfamily: ***Muntiacinae***

Genus: ***Muntiacus***

Muntiacus muntjak vaginalis (Indian muntjac; 2n=6/7; 800 bp)

Muntiacus reevesi (Chinese muntjac; 2n=46; 800 bp)

Subfamily: ***Cervinae***

Genus: ***Cervus***

Cervus elaphus canadenis (North American elk; 2n=68; 800 bp)

Subfamily: ***Odocoileinae***

Genus: ***Odocoileus***

Odocoileus hemionus (mule deer; 2n= 70; 1000 bp)

Odocoileus virginianus (white-tailed deer; 2n=70; 1000 bp)

Genus: ***Rangifer***

Rangifer tarandus caribou (woodland caribou; 2n=70; 1000 bp)

Genus: ***Capreolus***

Capreolus capreolus capreolus (European roe; 2n=70; 2000 bp)

Capreolus capreolus pygargus (Siberian roe; 2n=76-80; 1000 bp)

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